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Name of Candidate: Amy Starosciak
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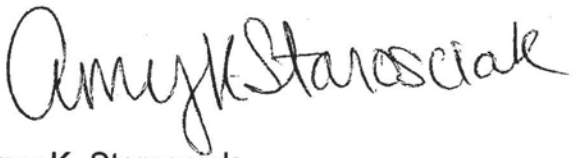

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Abstract

Title of Thesis: Effects of stress and social enrichment on alcohol intake, biological and psychological stress responses in rats

Author: Amy K. Starosciak, Doctor of Philosophy, 2010

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Addiction and drug abuse are pervasive in society, and can result in illness, legal and financial trouble, and even death for dependent users. Licit substances, including alcohol, are widely available, easily obtainable, and relatively inexpensive. More than 75% of Americans have used alcohol at least once in their lifetime, and more than half of adults are current drinkers. Unlike most other drugs, alcohol may be health-promoting in low to moderate doses. Excessive alcohol intake can cause serious immediate and long-term consequences to the drinker and to others. There is substantial anecdotal, clinical, and some experimental evidence that environmental factors, especially stress and social environment, affect alcohol self-administration. Stress often is associated with increased alcohol consumption. Social enrichment decreases drug self-administration, but there is mixed evidence regarding social enrichment and alcohol intake. These relationships lack experimental examination and the mechanisms underlying these relationships are not clear. The purpose of this research project was to examine experimentally the individual and combined effects of stress and social enrichment on alcohol self-administration (in two-bottle choice and operant self-administration paradigms) and alcohol's effects on

dopaminergic and serotonergic responses in the brain (nucleus accumbens, ventral tegmental area, prefrontal cortex) and on psychological constructs (anxiety via open field center time and depression via forced swim test) in male Wistar rats. The major hypotheses were: (1) stress would increase alcohol consumption and increase alcohol's effects on the stress response; (2) alcohol would decrease the biological and attenuate the psychological stress response; and (3) social enrichment would decrease alcohol consumption, attenuate the stress response, and attenuate alcohol's effects on the stress response.

Hypotheses 2 and 3 were partially confirmed. The major findings of this experiment were: (1) stress is biologically and behaviorally disruptive; (2) alcohol is disruptive; (3) stress and alcohol seem to cancel each other out on the forced swim test; and (4) social housing alters brain neurochemistry and behavior. These findings were consistent across many of the behavioral variables. In addition, this experiment demonstrates the value of combining behavior analyses with neurochemical analyses to gain a better understanding of how neurobiology might underlie behavior.

Effects of stress and social enrichment on alcohol intake, biological and psychological stress responses in rats

by

Amy K. Starosciak

Doctoral Dissertation submitted to the Faculty of the Neuroscience Graduate Program of the Uniformed Services University of the Health Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, 2010

Acknowledgements

When I was in elementary school, I was accepted into the East Brunswick School District's Talented and Gifted (TAG) program, where the students were taken out of the normal classroom several times a week for an hour or two and allowed to work on special projects, learn advanced material, and interact with peers at the same scholastic level. As my mother loves to tell and retell, I was extremely distressed by the fact that I was missing my fifth grade science class to attend TAG classes. I begged her to let me quit and she did. My teachers were upset, my TAG teacher was upset, and the superintendent of schools was upset. Didn't I know I was being afforded an exceptional opportunity to develop and hone special academic skills? In my 10-year old mind, all I knew was that I always missed science class; I did not want to miss that class anymore! To this day, I am still the only student in East Brunswick to have dropped out of the TAG program (though I returned to the class the following year, when it was a separate class that did not overlap with other classes). At that time, my mother knew that science was the subject for me, and that I would succeed in science in the future. I believe that that experience was the first step in the many steps that brought me to where I am today.

The last five years have been ruthless and enduring, but I am grateful for the many people who have helped me, guided me, supported me, pushed me, laughed with me, cried with me, and in some cases, carried me. First, I would like to thank the outstanding Uniformed Services University support staff in the Graduate Education Office and Department of Laboratory Animal Medicine, as

well as the faculty (especially Drs. Zygmunt Galdzicki and Denes Agoston for providing me with funding in the last year), staff and students of the Neuroscience Program and Department of Medical and Clinical Psychology. I also thank the members of my doctoral dissertation committee (Drs. Greg Mueller, Maria Braga, Andrew Waters, and Lei Zhang) for their guidance throughout the many steps involved in the development and completion of my doctoral research.

Through my various lab rotations and collaborations with different faculty members in the Neuroscience Program, I have learned many techniques and skills that have helped me to become a more well-rounded neuroscientist, but I am especially grateful to Dr. David Jacobowitz. It was an honor and a privilege to work with such a renowned neuroanatomist and neuropharmacologist. He was always extremely patient when teaching me new techniques, but never treated me as a "kid," even though I am the same age as his granddaughter. I am also grateful for the opportunity to work with Dr. Mike Baumann at the National Institute on Drug Abuse. Without the willingness to share his neurochemical expertise and his laboratory, I would not have been able to complete my doctoral research.

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from the rest, Kristen Hamilton. Kristen and I have been together since I started school 5 years ago. We've been on the same track (though in different programs) since the beginning, and she has been there for me since Day 1. Though there were times when we have butted heads, she has been more than a friend, a sister. From the wacky times in Puerto Rico, to the stressful times of running our dissertation experiments, Kristen never left my side, and never let me fall too far. Kristen, I cannot thank you enough for the love and encouragement you have given me.

Outside of the lab, my friends (the USU crew, the UMBC crew, the Pickles crew, and the Jersey crew) and family have shown me nothing but love and support during the most stressful of times. They remind me of what is important in life, and help keep me grounded while I strive to reach my goals. I am blessed to have such an amazing social foundation. Out of this strong network of friends and family, there are four people who were vital to my success in graduate school. The first is my advisor and mentor, Dr. Neil Grunberg. He has described me as an emotional pillar for the lab, always staying cool, calm and collected, but he has been my emotional pillar. He could always tell when something was bothering me, and always helped me find the best solution. Aside from the emotional support, Neil has pushed me to my maximum capabilities, and further, knowing that it would make me a better, stronger scientist and person. Neil comes from a long line of famous and extraordinary scientists, and I am grateful to have this in my "blood" and to continue the lineage. For this I will be forever in his debt. The second two people are my mother and father. They have always

inspired me to pursue my dreams in science by letting me drop out of TAG so I could attend science class, buying me my first microscope, taking me to museums and science centers, and passing around my first published paper to the whole family and their friends. Never once have they doubted my abilities. Thank you for embracing my scientific aspirations. The last, and most important, person is my boyfriend, Anthony. We've been through the worst of times at the beginning of graduate school, and it has only been getting better. He makes me laugh, keeps me motivated, and put his own career on hold while I finished school. For his love and support, I am eternally grateful. I look forward to continuing our journey in Miami, and wherever my career or his career takes us.

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Introduction

There is considerable evidence that environment affects drug self-administration (including alcohol). Environmental variables include stress and social environment. Stress often increases drug self-administration (including alcohol self-administration) and may augment or enhance drug effects (*e.g.,* Piazza and Le Moal, 1996; Goeders, 2003; Kassel et al., 2003; Sinha, 2007; Koob, 2008; Sinha, 2008; Grunberg et al., 2010). Social enrichment often decreases drug self-administration and may decrease drug effects (*e.g.,* Bardo et al., 1995; Bardo et al., 2001). The individual effects of these variables have been reported frequently in the preclinical (animal) and clinical (human) literature, and as anecdotal (human) accounts. However, few reports rigorously examined the combination of stress and social enrichment on alcohol consumption (self-administration), and relevant biological mechanisms and psychological variables in animals. This experiment was designed to: (1) examine effects of stress and social enrichment on alcohol consumption; and (2) to measure dopamine, serotonin, and their major metabolites in regions of the brain associated with reward and reinforcement (*i.e.,* nucleus accumbens, ventral tegmental area, prefrontal cortex) in rats. The first purpose is important because effects of stress and social enrichment on alcohol consumption have never been examined in a full-factorial design so that individual and combined effects could be clearly delineated. With regard to the second purpose, measuring neurotransmitter levels (specifically those related to reward and mood) in these brain regions after administration of stress (or no stress), social enrichment (or isolation), and

alcohol (or water) has not been done before. The neurochemical results from this experiment will therefore add to the current understanding of neurochemical mechanisms involved in stress, enrichment, and alcohol consumption.

The relevant literature regarding alcohol self-administration, stress, and environmental enrichment is reviewed below. An emphasis is placed on animal models because rats were used in the current experiment. Within each of these sections, underlying neurobiology and neurochemistry of these topics is discussed. Then, the three variables of alcohol self-administration, stress, and social environment are considered together. After introducing these topics, the detailed methodology for the experiment is described, the results are presented and then discussed in the context of the current literature.

Alcohol Self-Administration

More than 3/4 of Americans have a lifetime prevalence of alcohol use (SAMHSA, 2002), and more than 1/2 of adults aged 21 and older are current drinkers (*i.e.,* have had at least one drink in the preceding 30 days) (SAMHSA, 2007). Perhaps atypical from other drugs, alcohol can be beneficial to health in low to moderate doses (Corrao et al., 2000; Corrao et al., 2004; Kloner and Rezkalla, 2007; Djousse and Gaziano, 2008; Costanzo et al., 2010; Hvidtfeldt et al., 2010); however, excessive alcohol use in the form of binge drinking (5 or more drinks for men; 4 or more drinks for women) and heavy drinking (> 2 drinks/day for males; >1 drink/day for females) can cause serious immediate and long-term health and behavioral consequences (NIAAA, 2004; CDC, 2008).

Approximately 15% of American adults binge drink and 5% are heavy drinkers (NIAAA, 2006; CDC, 2007). Though these numbers were relatively stable through the 1990s, these destructive drinking patterns are increasing, especially in the young adult (ages 21-25) population (NIAAA, 2006; CDC, 2007). Alcohol use is the third leading cause of preventable death in the United States, resulting in over 75,000 deaths annually (CDC, 2008), of which 1/5 are caused specifically by alcohol-related car accidents (NCS, 2006). It is clear that excessive alcohol use can be dangerous, but it is not obvious how environmental factors such as stress and social enrichment might act separately or interact to affect alcohol intake. It is logistically and ethically impossible to conduct true, causal experiments in humans. Animal models provide a way to manipulate and causally test effects of stress and enrichment on alcohol self-administration with a great degree of control.

Animal Models of Alcohol Administration

Although studying stress and social environment on alcohol consumption in humans is ideal, it is difficult to perform controlled and causal experiments on these topics. For example, social environment is highly variable from person to person and difficult to quantify, qualify, and control (*e.g.,* number of relationships, quality of relationships, method of interaction, etc.). There are also ethical and legal considerations with regard to administering a potentially addictive drug (such as alcohol) to minors and young adults. For the present research, a rat model was used.

In laboratory research models of alcohol's effects on the brain, body, and behavior, there are several methods for the experimenter to administer alcohol, and several methods of alcohol self-administration. Alcohol can be fed to laboratory animals either in the liquid or solid diet, administered through an intragastric feeding tube, injected subcutaneously or intraperitoneally, or inhaled as alcohol vapor (Tabakoff and Hoffman, 2000). While these methods offer controlled administration to examine effects of different alcohol doses on behavior and physiology, one cannot assess how much an animal actually drinks. Alcohol self-administration paradigms in rodents provide face valid models of alcohol consumption. A common method of self-administration is the two-bottle choice (2BC) paradigm, in which the animal has access to a bottle of alcohol solution, and a bottle filled with another fluid for 24 hours, in a limited access paradigm (*e.g.,* for 1 hour), or restricted to post-meal (prandial) drinking (Deatherage, 1972; Kulkosky, 1980; Linseman, 1987; Wolffgramm, 1990; Tabakoff and Hoffman, 2000; Rodd et al., 2004). The current experiment used the 24 hour two-bottle choice paradigm to precisely measure volume of alcohol consumed, and alcohol preference (in comparison to water). However, the two-bottle choice model has two limitations: (1) motivation to obtain alcohol cannot be assessed; (2) individual consumption patterns cannot be assessed for the animals in group housing. A second method of alcohol self-administration in the form of operant conditioning was necessary to address these limitations.

Operant conditioning models of alcohol self-administration, in which subjects learn to press a lever to receive a specified amount of alcohol (*e.g.,*

orally, intragastrically, intracranially), provide information about behavior and motivation. By using an operant paradigm and increasing the number of times the animal needs to press the lever to obtain the alcohol, one can assess motivation and consumption (Le et al., 1998; Le et al., 2000; Tabakoff and Hoffman, 2000; Samson and Czachowski, 2003; Gonzales et al., 2004). Further, there is evidence that animals self-administering alcohol in a free-choice paradigm also self-administer in operant conditions (Green and Grahame, 2008), and the free-choice paradigm is often used to elicit alcohol self-administration in the operant paradigm (Le et al., 1998; Le et al., 2000). These models have face validity because the animals can orally self-administer alcohol like humans and have predictive validity when comparing to the human situation (Tabakoff and Hoffman, 2000). By using the two types of alcohol self-administration paradigms, precise volumes of alcohol consumption as well as motivation to consume alcohol can be measured. Once alcohol (or other commonly abused drugs) is self-administered, profound effects can be found in the brain. In fact, drug and alcohol self-administration is mediated by underlying central neurobiological and neurochemical mechanisms within the “reward” pathway of the brain which are described in the following section.

Drug Reinforcement

A reward is a stimulus that increases the probability that a behavior will occur again by eliciting a pleasurable effect (Anselme, 2009). This process is also known as positive reinforcement. Natural rewards, including food and sex,

activate the mesolimbic dopamine system (often referred to as the “reward pathway”) which includes the ventral tegmental area (VTA), nucleus accumbens (NAc), septum, hippocampus, and amygdala (Feldman et al., 1997; Gonzales et al., 2004; Anselme, 2009). Drugs and alcohol also can be rewarding because they activate the mesolimbic dopamine system. One of the most thoroughly studied theories of drug action on reward pathways suggests that addictive drugs facilitate dopaminergic transmission in the mesolimbic dopamine system, either through direct stimulation of dopamine release from the VTA to the NAc (*e.g.,* cocaine, amphetamines) or disinhibition of dopamine release (*e.g.,* alcohol, barbiturates) (Gonzales et al., 2004; Koob and Le Moal, 2006a). More recent data suggest that the mesolimbic dopamine system also serves to strengthen the association of stimuli that predict reward and establish response behaviors that lead to drug addiction (a behavior pattern of psychoactive drug use characterized by overwhelming involvement with use of the drug, securing its supply, and a high likelihood to relapse to drug use after abstaining from use) and dependence (highly controlled or compulsive use of a psychoactive drug that is reinforced by the effects of that drug) (Brunton et al., 2006; Koob and Le Moal, 2006a). In addition, there are differences between the two areas of the NAc, the shell and the core. It seems that the NAc shell may be more involved in the initiation of drug-seeking behavior, whereas the NAc core may be more important in the maintenance of drug-seeking (Gonzales et al., 2004). The role of alcohol, as a rewarding drug that activates the mesolimbic dopamine system and interacts with serotonergic systems, is described in greater detail below.

Along with the mesolimbic dopamine reward pathway, serotonergic mechanisms have been implicated in alcohol self-administration as well. In fact, the serotonin system is more complex with regard to alcohol self-administration. Alcohol consumption may increase or decrease depending on the serotonin receptor subtype that is activated (Sellers et al., 1992a; Sellers et al., 1992b; Koob and Le Moal, 2006b; Iverson et al., 2009a). Further, serotonin is implicated in modulating mood (Iverson et al., 2009b) and depression; depressive-like behavior was measured in the current experiment.

Alcohol Reinforcement

Human and rat studies have reported that dopamine is released from the VTA to the NAc in response to many drugs, including alcohol. Because of the logistics involved with an *in vivo* analysis of ethanol-induced dopamine (DA) release in the human brain, limited data are available, and few reports are in the literature. [^{11}C]raclopride positron emission tomography (PET) in humans revealed that DA release in the NAc increases in response to alcohol consumption (Boileau et al., 2003). Rodent studies have provided much more detailed information regarding ethanol-induced DA release. Ethanol induces the release of DA from VTA neurons via direct (without input from surrounding neurons) (Brodie et al., 1999) and indirect (with input from nearby serotonergic neurons) (Campbell et al., 1996) mechanisms. Further, microdialysis studies indicate that when ethanol is intraperitoneally (i.p.) injected, DA increases in the NAc of several rat strains, including Long-Evans (Samson et al., 1997), Wistar

(Yoshimoto et al., 1991; Samson et al., 1997; Smith and Weiss, 1999), Sprague-Dawley (Yim and Gonzales, 2000), and rats bred to prefer ethanol (Smith and Weiss, 1999) or rats bred to be sensitive to ethanol (Piepponen et al., 2002). Local intracranial administration of ethanol into the NAc results in no change in accumbal DA release using voltammetry (Samson et al., 1997) or an increase in accumbal DA release using microdialysis (Yoshimoto et al., 1991; Lof et al., 2007), both in Wistar rats. Part of this discrepancy lies within the methods themselves.

Whereas the microdialysis technique measures slow changes in extracellular DA (which may be the result of DA release, metabolism, transport, reuptake, synthesis, and/or inhibitory control), voltammetry examines evoked DA release after stimulation (Budygin et al., 2001). Reports in the literature are mixed regarding the mechanism underlying ethanol reinforcement. This inconsistency seems to be the result of differences in neurochemical techniques, method of alcohol administration, or self-administration. The present experiment adds to this literature, and may add clarification by using an *in vitro* technique to examine levels of dopamine, also serotonin, and both their metabolites (Baumann et al., 1998; Baumann et al., 2005; Baumann et al., 2008) in microdissected VTA, NAc, and PFC of *post mortem* brain tissue (Shohami et al., 1983), so that individual neurochemicals can be examined, as well as dopamine and serotonin turnover in these brain regions. Further, this approach allows for complex, detailed behavioral analysis to accompany the neurochemical analysis,

an outcome that is difficult to design and execute with microdialysis and voltammetric studies.

Stress

Alcohol and drug use may be altered by a variety of environmental factors, including stress. Stress is pervasive in society and is experienced by nearly everyone. In fact, approximately 3/4 of Americans report some stress in their daily lives, and 2/5 of Americans report frequent stress (Carroll, 2007). Selye (1936) defined stress as the body's non-specific response to a particular demand, which, if left unrelieved, could become a pathological state. It is a process by which an organism can no longer adapt to the environmental pressures upon which the organism is subjected (Baum et al., 1997). A more recent conceptualization of stress was developed by McEwen (1998, 2000) who emphasizes that stress is the result of psychological and physiological processes, that in the short-term stress can be protective, but which, in the long-term or chronic stress, can be potentially delibitating. McEwen termed the short-term protective action of stress "allostasis" and the long-term problems associated with stress "allostatic load." Such long term stress effects include an increased risk for disease development (Cohen et al., 1995), and alcohol use and abuse (Piazza and LeMoal, 1998; Goeders, 2003) resulting from the inability to cope with real or perceived, psychological or physical demands. These definitions of stress are quite broad. In the context of the current experiment,

stress was operationalized as the exposure of an animal to a non-painful, aversive environment (Piazza and LeMoal, 1998).

Neurochemical and Neurobiological Changes to Stress

Stress elicits responses from two major chemical systems within the body. There is a rapid response from the sympathetic division of the autonomic nervous system in which norepinephrine is released from postganglionic sympathetic neurons and epinephrine is released from the adrenal medulla (Guyton and Hall, 2000a; Iverson et al., 2000). A slower endocrinological response to stress occurs via the hypothalamic-pituitary-adrenal (HPA) axis, in which corticotropin releasing factor (CRF) is released from the hypothalamus, signaling the anterior pituitary gland to release adrenocorticotropic hormone (ACTH), which in turn stimulates the release of cortisol (corticosterone in rodents) from the adrenal gland cortex (Guyton and Hall, 2000b). Higher levels of the catecholamines norepinephrine and dopamine occur in many brain regions, including the medial preoptic nucleus, paraventricular nucleus, median eminence, periventricular nucleus, arcuate nucleus (Moyer et al., 1978), forebrain (Richardson, 1984), and prefrontal cortex (Deutch et al., 1990; Deutch and Roth, 1990) in response to different stressors. Stress increases levels of serotonin release in the amygdala (Mo et al., 2008) and prefrontal cortex (Meloni et al., 2008), and serotonin may modulate the physiological stress response through 5-HT_{2C} receptor activation (Heisler et al., 2007). Though any of these hormones and neurochemicals can be used as biomarkers of stress, corticosterone in the

blood is the most commonly used as biomarker of stress (Kant et al., 1986; Meaney et al., 1991; Brown and Grunberg, 1995; Kant et al., 2001; Faraday, 2002; McEwen, 2008; Berger, 2009; Perry, 2009; Hamilton, 2010; Long, 2010).

Stress and Substance Use

Stress is associated with drug self-administration. People often use drugs to self-medicate or cope with stress (Khantzian, 1985; Sher, 2007; Grunberg et al., 2010), and people with substance use disorders often experience increased stress in response to various stressors (Koob and LeMoal, 1997; Brady and Sinha, 2005). Further, people who are drug-dependent tend to have increased drug cravings when they are under stress, and abstinent drug users have a higher rate of relapse when experiencing stress (Brady and Sinha, 2005; Breese et al., 2005). These effects of stress on drug use have been established in the human literature for many drugs, including alcohol (Ahola et al., 2006; Helzer et al., 2006; Schroder and Perrine, 2007), and some research suggests that chronic users of alcohol have an activated or stimulated HPA axis (Kreek and Koob, 1998). In human research, it is difficult to determine non-stressed baseline or pre-stress levels of the biological and psychological stress response because people tend to experience stress on a daily basis. True experiments using animal models have provided substantial evidence to support the human observation that stress is associated with drug or alcohol self-administration.

In rodent models, stress can induce drug self-administration, maintain or increase self-administration, induce re-instatement of self-administration after

abstinence, lengthen withdrawal from the drug, and worsen withdrawal symptoms (Grunberg et al., 2010). These effects of stress have been reported for cocaine (Erb et al., 1996; Ahmed and Koob, 1997; Piazza and LeMoal, 1998; Marquardt et al., 2004), amphetamines (Piazza and LeMoal, 1998), opiates (Shaham et al., 1996; Piazza and LeMoal, 1998; Lu et al., 2003; Kreek, 2007), nicotine (Schachter et al., 1977b; Schachter et al., 1977a; Pomerleau and Pomerleau, 1987; Buczek et al., 1999; Kassel et al., 2003; Grunberg and Shafer, 2005), and alcohol (Breese et al., 2005; Hansson et al., 2006; Fullgrabe et al., 2007; Sher et al., 2007). These results suggest that stress may cause a person to initiate and maintain drug use, prevent or inhibit drug abstinence, and/or induce relapse to drug use.

Stress and Alcohol Use

Several potential mechanisms exist that may cause an increase in alcohol self-administration during stressful periods. Because stress increases arousal, alcohol (a CNS depressant) may be used to self-medicate to decrease arousal caused by stress. Conger (1956) proposed that alcohol may serve to reduce stress-induced CNS arousal and stress-induced anxiety. It is important to appreciate that the correlation between stress and alcohol consumption may result from alcohol intake reducing the stress response, or from stress increasing alcohol intake (but not reducing stress). In other words, it is **not clear if alcohol consumption actually reduces the physiological and psychological stress responses** (Sher et al., 2007). Sher and colleagues (2007) also report that

attention is an important mediator and moderator in the relationship between alcohol and stress, such that alcohol reduces attention to the stressor, thereby reducing perceived stress. Neurobiological evidence suggests that stress and the resulting higher levels of glucocorticoids increase dopamine release in the mesolimbic dopamine system, and to sensitize this system to drug-induced dopamine release, thereby making the drug more rewarding (Piazza and LeMoal, 1998). This effect is well documented for opiates and psychostimulants (Kreek and Koob, 1998; Piazza and LeMoal, 1998; Goeders, 2003; Kreek, 2007). For the alcohol-stress relationship, there seems to be a combination of underlying psychological and neurobiological mechanisms, but these mechanisms are not well understood. In the current experiment, an animal model was used to determine whether stress increases alcohol consumption, and whether alcohol increases the physiologic (serum corticosterone) and attenuates the psychologic (anxiety and depression) indices of stress.

Stress Management

There are several ways to manage stress, in both everyday life and in the clinic. Coping is a process of managing demands that are appraised as taxing or exceeding the individual's resources, whereas stress management techniques can be taught to patients for managing stress-related autonomic arousal, muscular tension, and emotional distress with the goal of alleviating or controlling a distressing physical or psychological state (Benson et al., 1974; Mandle et al., 1996; Deckro et al., 2002). Seeking social support (the feeling that one belongs

to a social network) during stressful times is a way people often learn to cope with stress, and being with others may serve to buffer effects of stress (Cohen and Wills, 1985; Cohen et al., 1986; Baum and Posluszny, 1999). In animals, a situation analogous to social support is social enrichment, a form of environmental enrichment. The current experiment causally examined how social enrichment affected the stress response.

Environmental Enrichment

Environmental enrichment is generally comprised of the presence of physical objects with which to interact, and/or the presence of similar others (*e.g.,* members of the same species). Various animal studies (Wiesel and Hubel, 1963; Ruppenthal et al., 1976; Meaney et al., 1991; Fernandez-Teruel et al., 1997; van Praag et al., 2000; Elliott and Grunberg, 2005; Shafer, 2005; Singer et al., 2005; Tomchesson, 2005; Nithianantharajah and Hannan, 2006; Long, 2010) using impoverished or enriched environments have examined the mechanisms by which enrichment affects health, well-being, and development. Results from these animal studies have mirrored the evidence from clinical studies suggesting that poor social environments, or socially isolated environments can have long-term adverse health consequences on humans (Kaler and Freeman, 1994; Caspi et al., 2006; Danese et al., 2007). In the context of the current experiment, the social environment was manipulated (rats housed in groups of three or individually) to determine whether the presence of others reduces alcohol

consumption and attenuates the stress response, and to determine whether social environment can produce chemical changes in the brain.

Historical Context of Environmental Enrichment

The observation of environmental enrichment goes back over 100 years, and the systematic experimental manipulation of enriched environments began approximately 50 years ago. Darwin (1874) observed that the brains of domestic rabbits were considerably reduced in size when compared to the brains of wild rabbits. Darwin surmised that the domestic animals did not use their instincts and senses to their full capacity (Darwin, 1874). In 1947, Hebb described how laboratory rats that he had taken home for his children to play with performed better than rats that remained in the laboratory on a variety of learning tasks (Hebb, 1947). Similar results were reported with dogs (Clarke et al., 1951). In fact, Hebb suggested that brain morphological and physiological changes, now known as neuroplasticity, underlies the observed functional change (Hebb, 1947). In the 1960s, the first controlled studies in animals demonstrated that enriching an animal's environment could change or alter the animal's neuroanatomy and neurochemistry. Wiesel and Hubel (1963) described how visual deprivation altered the structure and function of brain regions responsive to visual stimuli in cats (Wiesel and Hubel, 1963). Other investigators examining enriched, rather than impoverished, environments reported that the morphological, cellular, and molecular changes in the brain enhanced learning

and memory in rats, and these results were similar in young, middle-aged, and older rats (Bennett et al., 1964; Diamond et al., 1964; Diamond et al., 1966).

Neurobiological and Neurochemical Changes to Enrichment

Enriched environments present at different times during an organism's life can have profound effects on brain neuroendocrinology, neuroanatomy, and neurochemistry. In the earliest experiments by Rosenzweig and colleagues, general brain changes had been found, including an increase in total brain weight (Bennett et al., 1969), increased cortical thickness (Bennett et al., 1964; Diamond et al., 1966), and changes in total DNA, RNA, and protein content (Rosenzweig and Bennett, 1969) in the brains of animals subjected to enriched environments. Enrichment can increase gliogenesis, neurogenesis, increase dendritic branching, enhance long-term potentiation amplitude and excitatory post-synaptic potentials, alter neurotransmitter receptor expression and receptor binding in different areas of the brain (Diamond et al., 1966; Berman et al., 1996; van Praag et al., 2000; Hellemans et al., 2005; Zhu et al., 2005; Rahman and Bardo, 2008). Further, enrichment in the form of animal handling during the early postnatal period increases hippocampal glucocorticoid binding in the hippocampus and enhances responsiveness of the HPA axis (Meaney et al., 1991). Enrichment has been reported to attenuate age-related declines in learning and memory, and can beneficially alter the progression of neurological (*e.g.,* Alzheimer's disease, traumatic brain injury, stroke, epilepsy, etc.) and psychiatric (*e.g.,* major depressive disorder, bipolar disorder, schizophrenia,

substance use disorders, etc.) disorders (Nithianantharajah and Hannan, 2006). Clearly, environmental enrichment can have substantial effects on brain morphology, physiology, and chemistry. There is some evidence to support effects of environmental enrichment (or deprivation) to increase striatal dopamine (Brenes and Fornaguera, 2008; Brenes et al., 2008) and serotonin in the prefrontal cortex (Brenes et al., 2008), but reports in the literature are few. The current experiment was designed to further explore how social enrichment specifically affects brain neurochemistry by examining levels of catecholamines and indoleamines in specific brain regions.

Enrichment and Substance Use

Although controlled experiments examining the effects of environmental enrichment began with Rosenzweig's group in the 1960s, little attention was given to the effect of enriched environments on drug action for several decades. In the 1990s, Bardo and colleagues began studying the effects of enrichment on effects of amphetamine (Bowling et al., 1993; Bowling and Bardo, 1994; Bardo et al., 1995), and since then there have been numerous studies examining the effects of enrichment on many drugs, including amphetamine (Zimmerberg and Brett, 1992; Bardo et al., 2001; Stairs et al., 2006; Fukushiro et al., 2007), cocaine (Solinas et al., 2008), nicotine (Grunberg et al., unpublished data), barbital (Zimmerberg and Brett, 1992), and opiates (Smith et al., 2003; Smith et al., 2005; Yang et al., 2006; Xu et al., 2007), with the consensus that enrichment decreases drug self-administration and/or attenuates drug effects in the brain

and on behavior. However, the reports in the literature have conflicting findings regarding environmental enrichment, alcohol self-administration, and alcohol's effects which are reviewed in the following section.

Enrichment and Alcohol Use

While there is a general agreement that environmental enrichment decreases self-administration of amphetamines, cocaine, nicotine, barbital, and opiates, the literature on the effects of enrichment on alcohol self-administration is not well established. Similar to the effects of environmental enrichment on other drugs, several reports indicate that environmental enrichment decreases alcohol self-administration (Deatherage, 1972; Parker and Radow, 1974; Schenk et al., 1990; Wolffgramm, 1990; Deehan et al., 2007) or decreases alcohol's effects on cognition and behavior (Hellemans et al., 2005). However, other groups have reported the opposite effect – that environmental enrichment increases alcohol self-administration (Rockman et al., 1986; Rockman and Gibson, 1992; Fernandez-Teruel et al., 2002). The most obvious explanation for these different findings is the type of environmental enrichment used and the timing of that enrichment. In the reports where enrichment decreases self-administration, the animals were living in their respective environments during behavior testing (Deatherage, 1972; Parker and Radow, 1974; Schenk et al., 1990; Hellemans et al., 2005; Deehan et al., 2007). In the other experiments, animals were exposed to early enrichment, but not to enrichment during behavior testing when alcohol self-administration was increased (Rockman et al., 1986;

Fernandez-Teruel et al., 2002). By taking animals out of an enriched environment, a stressful situation may have been induced that increased alcohol consumption. This response may be especially true for animals that were exposed to social enrichment, as opposed to just physical enrichment. It is clear that both stress and environment each individually play a role in alcohol self-administration, and that the pharmacological actions of alcohol involve dopamine and the reward pathway in the brain. However, there have not been any reports in the literature that examine all three variables in one experiment. The next section focuses on bringing all of these variables together.

Alcohol, Stress, Enrichment, and Reward

There is evidence that stress increases DA release in the PFC (Finlay et al., 1995; Del Arco and Mora, 2001; Nanni et al., 2003; Pascucci et al., 2007) which, in turn, inhibits DA release in the NAc (Nanni et al., 2003; Pascucci et al., 2007). Subsequently, a DA-releasing drug may be self-administered to offset this inhibited DA release. This idea is consistent with the fact that stress is associated with an increase in drug use (including alcohol) (Ahola et al., 2006; Helzer et al., 2006; Schroder and Perrine, 2007), and that these drugs increase the release of DA in the NAc (Gonzales et al., 2004; Koob and Le Moal, 2006a). In other words, stress may decrease accumbal dopamine release (and decrease reward) so that a drug user may self-medicate (Khantzian, 1985; Sher et al., 2007) with a drug of choice to increase accumbal DA and decrease feelings of stress or discomfort (as a form of negative reinforcement).

Some evidence also exists suggesting that accumbal DA release increases in response to confinement stress (Wu et al., 1999), or stress mimicked by the oral or intravenous administration of corticosterone (Piazza et al., 1996). Voltammetric studies have reported similar results, and also indicate that γ-amino butyric acid (GABA) in the PFC (Doherty and Gratton, 1999) and DA in the basolateral amygdala (Stevenson and Gratton, 2003) may modulate the accumbal DA stress response. In terms of reinforcement and drug use, if stress does increase accumbal DA, and drugs of abuse increase DA release in the NAc, then the stimuli associated with stress and the stimuli associated with drug use may become associated with each other, thereby increasing the likelihood of a person to use drugs when experiencing a stressful situation. However, one must be wary when coming to any conclusions based on the available evidence because there are considerable differences across all studies, including the type and duration of the stressor, the phase in which the stress and the DA analysis were performed (active/dark vs. inactive/light), and the strain of rat used. Therefore, it is difficult to determine the exact effects of stress on DA release in the NAc and more research is necessary to elucidate the mechanism.

Though there is considerably more research reported on the effects of stress on central dopamine and serotonin, there has been little research on effects of stress and environmental enrichment on central dopaminergic and serotoninergic systems. If environmental enrichment affects alcohol self-administration, then it may be via a dopaminergic or serotonergic mechanism in the brain reward pathway. Del Arco and colleagues (2007) reported that D1

receptor stimulation in the PFC through its cholinergic efferents may be involved in increased motor activity in rodents (Del Arco et al., 2007). Also, there is some evidence that environmental enrichment decreases stress-induced DA elevation in the PFC (Segovia et al., 2008), and increases an animal's ability to adapt to stress (Sullivan and Dufresne, 2006). However, little research attention has examined effects of stress and environmental enrichment on dopamine or its metabolites. If social enrichment indeed serves as a stress buffer, then this phenomenon should be reflected in the central neurochemistry relevant to alcohol self-administration.

At this time, the mechanisms underlying stress and enrichment effects on alcohol consumption are unknown. It may be that these factors are influencing central neurochemistry. The current research was designed to examine the individual and combined effects of alcohol, stress, and social enrichment on dopamine and serotonin in the VTA, NAc, and PFC in an attempt to elucidate underlying neurochemical mechanisms.

Hypotheses

The purpose of this doctoral dissertation research was to examine individual and combined effects of stress and social enrichment on alcohol consumption, and also to examine the effects of alcohol consumption, stress, and social enrichment on brain neurochemistry, anxiety, and depression in rats. Based on the current literature discussed in the preceding sections, it was hypothesized that: **(1) stress will** (a) increase alcohol self-administration

(Piazza and LeMoal, 1998; Goeders, 2003; Ahola et al., 2006; Helzer et al., 2006), (b) increase biological and psychological stress responses (Selye, 1936), and (c) increase effects of alcohol on biological and psychological stress responses (Conger, 1956; Sher et al., 2007); **(2) alcohol will** (a) increase biological stress responses (e.g., stress hormone levels) (Kreek and Koob, 1998) and (b) attenuate psychological stress responses (e.g., indices of anxiety) (Conger, 1956; Sher et al., 2007); and **(3) social enrichment will** (a) decrease alcohol self-administration (Deatherage, 1972; Parker and Radow, 1974; Schenk et al., 1990; Wolffgramm, 1990; Deehan et al., 2007), (b) attenuate biological and psychological stress responses (Singer et al., 2005; Yang et al., 2006), and (c) attenuate effects of alcohol on biological and psychological stress responses (Hellemans et al., 2005).

Methods

To address the above hypotheses, this experiment was a 2 (alcohol or no alcohol) x 2 (stress or no stress) x 2 (isolated vs. triple-housing) full-factorial design (see Table 1), with animals assigned to each condition based on random removal from shipping boxes at the start of the study (see Table 2 for Experimental Timeline). Animals (N=72; n=9 per experimental condition) were split into two overlapping cohorts (Cohorts A and B) of 36 animals, such that 3 or 6 animals from each of the 8 groups were represented in each cohort. This

cohort and grouping strategy was used for the logistical purposes to complete the behavior portion of this experiment.

Table 1 – Grouping Strategy for Independent Variables

Group	Alcohol	Stress	Housing	# Animals in Coh. A	# Animals in Coh. B
1	No	No	Isolated	6	3
2	No	No	Enriched	6	3
3	No	Yes	Isolated	3	6
4	No	Yes	Enriched	3	6
5	Yes	No	Isolated	6	3
6	Yes	No	Enriched	6	3
7	Yes	Yes	Isolated	3	6
8	Yes	Yes	Enriched	3	6

The dependent variables were biological and behavioral. The biological variables included levels of dopamine, serotonin, and their metabolites from VTA, NAc, and PFC tissue samples, serum alcohol content, serum corticosterone concentration (as a biochemical index of the stress response), and body weight. Behavioral variables were alcohol consumption in the two-bottle choice paradigm and in an operant conditioning paradigm (to assess volume of alcohol consumption and motivation to consume alcohol), open field locomotor activity (to assess general movement, exploratory behavior, and anxiety-related behavior), rotarod performance (to assess balance and coordination), immobility in the forced swim test (to assess the depression symptom of learned helplessness), and food and water consumption (which may affect alcohol intake). Animal husbandry conditions, independent variables, dependent variables, and experimental timeline are explained in detail below. Tables 1-13

appear in the text, Tables 14-87 (statistical tables) appear in Appendix A, and all figures appear in Appendix B.

Table 2 – Experimental Timeline

Week	Cohort A	Cohort B
1	Animals arrive, group assignment, gentling	---
2	0% 2BC, stress begins	---
3	3% 2BC, stress	---
4	6% 2BC, stress	---
5	12% 2BC, stress	---
6	LSA training	Animals arrive, group assignment, gentling
7	LSA training	0% 2BC, stress begins
8	3% LSA, stress	3% 2BC, stress
9	6% LSA, stress	6% 2BC, stress
10	12% LSA, stress, sacrifice	12% 2BC, stress
11	Brain slicing	LSA training
12	Brain slicing	LSA training
13	Brain micropunching	3% LSA, stress
14	Brain micropunching	6% LSA, stress
15	HPLC analyses	12% LSA, stress, sacrifice
16	HPLC analyses	Brain slicing
17	HPLC analyses	Brain slicing
18-19	---	Brain micropunching
20-22	---	HPLC analyses
23	Serum cort and EtOH assays	Serum cort and EtOH assays
24+	Analyzing data, interpreting results, writing dissertation	Analyzing data, interpreting results, writing dissertation

X % = Percentage of ethanol used
2BC = Two-bottle choice paradigm
LSA = Operant liquid self-administration paradigm
HPLC = High performance liquid chromatography
Cort = Corticosterone
EtOH = Ethanol

Animals and Housing

The animals used in this experiment were 72 male Wistar albino rats (Charles River Laboratories, Wilmington, MA) that were 38 days old at the beginning of the experiment. Only male animals were used in this study because males are more likely to drink alcohol than females, and because males binge

drink and heavy drink at higher rates, and are more likely to suffer adverse consequences to alcohol consumption (Naimi et al., 2003; CDC, 2008). Wistar rats were used because they are most often used in alcohol self-administration studies and are more likely to self-administer alcohol than other strains (Parker and Radow, 1974; Kulkosky, 1980; Rockman et al., 1986; Linseman, 1987; Wolffgramm, 1990; Rockman and Gibson, 1992; Weiss et al., 1993; Rodd et al., 2004; Hansson et al., 2006; Turyabahika-Thyen and Wolffgramm, 2006; Badia-Elder et al., 2007; Fullgrabe et al., 2007; Funk et al., 2007). Thirty-eight day old rats were chosen for this experiment because they are adolescents (Spear, 2000a; Spear, 2000b, 2002; Bell et al., 2006), would begin the alcohol regimen before adulthood is reached, and this time of life is often when people are first exposed to alcohol and are most likely to drink the heaviest (Naimi et al., 2003; CDC, 2008). The number was chosen because the experimental design consisted of 8 different conditions, with 9 rats per condition (see power estimates under Data Analytic Strategy, page 41). Rats were housed either individually in standard polycarbonate rat cages (42.5 x 20.5 x 20 cm; Figure 1a) or in groups of 3 in larger polycarbonate cages (46 x 36 x 20 cm; Figure 1b). Both groups had hardwood chip bedding (Pine-Dri) and continuous access to food (Harlan Teklad 4% Mouse/Rat Diet 7001) and water. The housing room was maintained on a 12 hour reverse light cycle (lights on at 1900h, so that animals could be behaviorally tested during their normal active or dark period), at room temperature and 50% relative humidity. Half of the animals in each condition were stressed, and half of the animals in each of these conditions had access to alcohol (see Table 1, page

22). All animal procedures were approved by the Uniformed Services University Institutional Animal Care and Use Committee, and were conducted in compliance with the National Institutes of Health rules and regulations for animal research.

Independent Variables

Ethanol Administration

Ethanol was self-administered in two ways: a two-bottle choice paradigm (2BC; 50-74 days old) and an operant liquid self-administration paradigm (LSA; 75-106 days old). To elicit ethanol self-administration, ethanol solution was available to the animals in their home cages, in addition to the water bottle, in the 2BC paradigm (Figure 2). The ethanol solutions were available for 24 hours per day, in increasing concentrations (3%, 6%, and 12% v/v) for 1 week per concentration (MacDonnall and Marcucella, 1979; Linseman, 1987). With this paradigm, animals are introduced to several concentrations of ethanol, and the volume consumed can be carefully monitored.

After ethanol self-administration was elicited, the motivation to seek ethanol was measured in the LSA paradigm (Le et al., 1998; Le et al., 2000) during the animals' dark or active phase. The LSA chambers (Med Associates, St. Albans, VT) consisted of 2 retractable levers and 2 retractable sipper tubes (Figure 3). During the training procedure, both levers were available to the animals, but only one lever activated a sipper tube of 3% ethanol solution to enter into the chamber. The other lever was inactive during training (*i.e.,* nothing

occurred when it was pressed). Animals were trained for a half hour daily for two weeks in this manner. Once the animals learned to lever-press for the ethanol, both levers became active during the three week testing phase. During this phase, the active lever continued to signal the ethanol sipper tube; however, the inactive lever became active and signaled a sipper tube of water. This slight change in procedure allowed for alcohol preference compared to water to be examined. Each week during this phase, animals had access to increasing concentrations (3%, 6%, and 12% v/v) for 1 week per concentration. After the conclusion of the 12% LSA, animals were again allowed 24-hour access to 12% EtOH in the 2BC paradigm for several days until euthanasia and collection of tissue specimens. Animals in the no ethanol condition received water instead of ethanol in both procedures. Both ethanol and no ethanol groups were included in this experiment to determine whether stress and enrichment actually affect alcohol self-administration, or whether these variables only alter overall liquid (water) and/or calorie (food) consumption, motor activity (horizontal locomotor activity), or exploratory activity (vertical locomotor activity).

There were several reasons for using the two methods. Precise volumes of ethanol consumption can be measured for each cage in 2BC, however, only average consumption can be measured for group-housed animals. With operant LSA, one can assess alcohol consumption (by number of licks on the bottle spout) and motivation to consume alcohol (by number of lever presses) by individual rats. In other words, group housed rats drink with their cage mates during the 2BC task (and isolated animals drink alone), and all animals drink

alone during the operant LSA task. Previous reports (Deatherage, 1972; Parker and Radow, 1974; Rockman et al., 1986; Schenk et al., 1990; Wolffgramm, 1990; Rockman and Gibson, 1992; Fernandez-Teruel et al., 2002; Deehan et al., 2007) that examined effects of environmental enrichment on alcohol consumption differed when enrichment occurred (either during alcohol consumption monitoring, or some time before alcohol consumption monitoring), which may explain differences in findings. Using the two paradigms was designed to evaluate alcohol self-administration more thoroughly.

Stress

This experiment combined a predator stressor with several unpredictable stressors to elicit a physiological and psychological stress response based on recent studies in our laboratory (Berger, 2009; Perry, 2009; Hamilton, 2010; Long, 2010). The paradigm consisted of exposing the animals to a small amount of predator (fox) urine (Hayley et al., 2001), unpredictable flashing lights, loud noises, and cage shaking. All of these stressors occurred in a standard polycarbonate mouse cage (28 x 17.5 x 12.5 cm) that was similar to, but smaller than the home cage. These procedures occurred in a similar room separate from the housing room in bright, white light.

To induce stress, the animals were transferred to a room different from the housing room, and individually placed into mouse cages separate from the home cage. A small quantity of synthetic fox urine (15 mL) was soaked onto a cotton ball and placed in each stress cage in various locations each day. Each day, a

different unpredictable stressor (*i.e.,* noise, light, cage shaking) was used in combination with the predator stress to reduce any habituation to the stressor (Tables 3 and 4). The entire procedure lasted 10 minutes. At the conclusion of the stress procedure, rats were returned to their home cages, and placed back into their housing room. The last time animals received stress was 3-4 days before euthanasia and collection of tissue specimens. Animals in the non-stress group were brought to a room separate from the housing room and the stress room, and handled briefly to ensure that any changes in the stressed group was the result of the stressors themselves, and not to extra experimenter handling. This particular stress method (combined predator stressor and unpredictable stressors) has been shown in our laboratory to produce reliable increases in anxiety-like behaviors and serum corticosterone levels (Berger, 2009; Perry, 2009; Hamilton, 2010; Long, 2010).

Table 3 – Detailed Stress Procedure, Cohort A

Stress Day	Cotton Ball Placement	Stressor
1	Front-right corner	Fox urine only
2	Front-left corner	Fox urine + overhead lights flashed 10x @ minute 2, 6, and 9
3	Back-right corner	Fox urine + metal banging for 10 sec @ minute 3, 5, and 8
4	Middle-left side	Fox urine + cage shake 10x @ minute 1, 5, and 9
5	Middle-right side	Fox urine only
6	Back-right corner	Fox urine + whistle blown 6x @ minute 2, 6, and 8
7	Back-left corner	Fox urine + coin shake 10x @ minute 3 and 7
8	Front-left corner	Fox urine + coin shake 10x @ minute 3 and 7
9	Back-left corner	Fox urine + overhead lights flashed 10x @ minute 2, 6, and 9
10	Front-right corner	Fox urine + coin shake 10x @ minute 3 and 7
11	Back-left corner	Fox urine + metal banging for 10 sec @ minute 3, 5, and 8
12	Middle-right side	Fox urine only + whistle blown 6x @ minute 2, 6, and 8
13	Middle-left side	Fox urine + cage shake 10x @ minute 1, 5, and 9
14	Front-left corner	Fox urine + cage shake 10x @ minute 1, 5, and 9
15	Front-right corner	Fox urine only
16	Back-right corner	Fox urine + metal banging for 10 sec @ minute 3, 5, and 8
17	Middle-left side	Fox urine + cage shake 10x @ minute 1, 5, and 9

Table 4 – Detailed Stress Procedure, Cohort B

Stress Day	Cotton Ball Placement	Stressor
1	Front-right corner	Fox urine + coin shake 10x @ minute 3 and 7
2	Back-left corner	Fox urine + metal banging for 10 sec @ minute 3, 5, and 8
3	Middle-right side	Fox urine only
4	Middle-left side	Fox urine + cage shake 10x @ minute 1, 5, and 9
5	Front-left corner	Fox urine + cage shake 10x @ minute 1, 5, and 9
6	Front-left corner	Fox urine + overhead lights flashed 10x @ minute 2, 6, and 9
7	Back-right corner	Fox urine + metal banging for 10 sec @ minute 3, 5, and 8
8	Middle-left side	Fox urine + cage shake 10x @ minute 1, 5, and 9
9	Back-right corner	Fox urine + coin shake 10x @ minute 3 and 7
10	Front-left corner	Fox urine + coin shake 10x @ minute 3 and 7
11	Back-left corner	Fox urine + overhead lights flashed 10x @ minute 2, 6, and 9
12	Front-right corner	Fox urine only + coin shake 10x @ minute 3 and 7
13	Back-left corner	Fox urine + metal banging for 10 sec @ minute 3, 5, and 8
14	Middle-right side	Fox urine + whistle blown 6x @ minute 2, 6, and 8
15	Middle-right side	Fox urine only
16	Middle-left side	Fox urine + cage shake 10x @ minute 1, 5, and 9
17	Front-left corner	Fox urine + cage shake 10x @ minute 1, 5, and 9

Biological Dependent Variables

Body Weight

Body weight was measured three times per week for the duration of the experiment using an electronic Sartorious balance that takes a series of 10 weight readings (in approximately 10 s) and then provides an average of these readings (Berger, 2009; Perry, 2009; Hamilton, 2010; Long, 2010). This method accounts for changes in weight displacement as the animal moves on the

balance. Body weight was measured 27 times during the course of the experiment. This measure was used to ensure that animals remained healthy throughout the experiment.

Tissue Collection

Trunk blood was collected after CO_2-anesthetized decapitation and briefly stored in capped 15 mL polypropylene centrifuge tubes on wet ice until centrifugation. Samples were spun at 5^{o}C for 20 minutes at 25,000 rpm to separate serum from the rest of the blood. Serum was pipetted into 1.5 mL Eppendorf tubes and stored at -80^{o}C (Faraday et al., 2005; Berger, 2009; Perry, 2009; Hamilton, 2010; Long, 2010) until further analysis of corticosterone and ethanol content.

After decapitation, brains were removed, placed into a Jacobowitz brain block, and sliced at the level of the cerebellum. The rostral portions of the brains were rapidly frozen using dry ice, and stored at -80^{o}C until slicing. A microtome inside a -6^{o}C Cryostat was used to slice the brains at 300 µm; slices were adhered to 5 x 7.5 cm glass slides by briefly thawing the slices on the slides, and refreezing the slides on dry ice. Slides were stored at -80^{o}C until microdissection of brain regions (Palkovits, 1973; Jacobowitz, 1974; Palkovits and Jacobowitz, 1974).

Tissue Microdissection

Relevant brain regions (VTA, NAC, PFC, see Figure 4) were microdissected with stainless steel cannulae (750-1000 μm diameter) over a -10^{o} to -5^{o}C coldplate (Palkovits, 1973; Jacobowitz, 1974; Palkovits and Jacobowitz, 1974) and placed in 50 μL of 0.1 N perchloric acid with 100 μM EDTA in a 1.5 mL Eppendorf tube. Samples were briefly (~2 sec) microfuged to bring the tissue into the solution. After micropunching the brain tissue, samples were sonicated (3 sec pulses, 3x in a Kontes micro-ultrasonic cell disrupter) and stored at -80^{o}C until protein assays were performed.

Protein Assay

Samples were thawed and protein concentrations were determined using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, CA), using bovine serum albumin standards (Bradford, 1976). The protein assay was necessary so that neurotransmitter levels could be standardized and expressed in terms of amount of protein in each brain region (Baumann et al., 1998). An increase in protein concentration of the sample is directly related to an increase in absorbance at 540 nm; samples were compared to a standard curve for calculation of protein concentrations. Fifty μL 0.1 N perchloric acid with 100 μM EDTA was added to the samples subsequent to the completion of the protein assay to ensure enough volume for high performance liquid chromatography (HPLC) analysis. Samples were stored at -80^{o}C until HPLC analysis (Baumann et al., 1998).

Neurotransmitter Levels

Neurochemical analysis of microdissected VTA, NAc, and PFC (Figure 4) was performed to measure NE, DA, 5-HT, and selected metabolites (DOPAC, HVA, 5-HIAA) via HPLC with electrochemical detection (Shohami et al., 1983; Baumann et al., 1998; Baumann et al., 2008). In short, brain tissue punches from individual rats were diluted in 100 µL of ice cold 0.1 N $HClO_4$ (described above) and homogenized by sonication. Homogenates were spun at 15,000 rpm for 15 min at a temperature of 4^{o}C. Aliquots (20 µL) of the resulting supernatant were injected onto a C18 reverse-phase octadecyl silane (ODS) HPLC column, linked to a coulometric detector (Environmental Sciences Associates, Bedford, MA). A mobile phase consisting of 50 mM Na_2HPO_4, 250 µM Na_2EDTA, 0.04% sodium octanesulfonic acid, and 25% methanol (pH=2.75) was recirculated at 0.9 mL/min. Neurotransmitter and metabolite concentrations were determined using a Millennium software system (Waters, Inc., Milford, MA) to compare peak heights of unknowns to those of standards. Standard curves were linear and reproducible over a range of 10-1000 pg for each analyte. The lower limit of detection was ~3 pg per injection. Raw neurotransmitter and metabolite data are expressed as pg per µg protein (Shohami et al., 1983; Baumann et al., 1998; Baumann et al., 2008).

Blood-Alcohol Concentration (BAC)

Serum BAC was determined using an enzymatic assay kit (NAD-ADH Reagent Multiple Test Vial, Sigma-Aldrich, St. Louis, MO). Alcohol

dehydrogenase catalyzes the oxidation of ethanol to acetaldehyde with the concurrent reduction of nicotinamide adenine dinucleotide (NAD). The reaction causes increased absorbance at 340 nm that is directly proportional to the concentration of ethanol in the sample (Poklis and Mackell, 1982; Webb et al., 2002; Liao et al., 2007). This measure was included to verify that animals in the ethanol group did indeed consume ethanol in the 2BC and the LSA paradigms and to determine whether stress or enrichment affected the levels of ethanol in the body.

Serum Corticosterone

Serum corticosterone levels were used as a biological marker of stress (Brown and Grunberg, 1995; Faraday, 2002; Berger, 2009; Perry, 2009; Hamilton, 2010; Long, 2010). An ImmunoChem Double Antibody radioimmunoassay (RIA) kit with radiolabeled ^{125}I-corticosterone (MP Biomedicals, Costa Mesa, CA) was used to measure the concentration of corticosterone in serum samples taken at the conclusion of the study. A gamma counter measured radioactivity in the samples as disintegrations per minute, which was then converted into concentrations of corticosterone in ng per mL of serum. This measure was included to verify that animals in the stressed condition did react to the chronic stressor with activation of the HPA axis, and to determine effects of enrichment and alcohol consumption on the biochemical stress response (Fahey and Cheng, 2008; Gutierrez-Mariscal et al., 2008; Berger, 2009; Perry, 2009; Hamilton, 2010; Long, 2010; Shin et al., 2010).

Behavioral Dependent Variables

Food and Water Consumption

Food and water consumption were measured three times per week for the duration of the experiment. On each measurement day, the amount of total food or water was measured and subtracted from the previous measurement to provide and value for amount consumed (Berger, 2009; Perry, 2009; Hamilton, 2010; Long, 2010). In total, 26 measurements of food consumption and 26 measurements of water consumption were made. Food and water consumption were measured to ensure that animals remained healthy over the entire experiment, and so that values could be covaried with ethanol consumption during statistical analyses, if necessary, because differences in food or water consumption may affect ethanol consumption.

Ethanol Self-Administration

Ethanol self-administration was assessed in two ways. To elicit ethanol self-administration, ethanol solution in a water bottle was made available to the animals in their home cages, next to the normal water bottle, in a 2BC paradigm (Figure 2). The ethanol solutions were available for 24 hours per day, in increasing concentrations (0%, 3%, 6%, and 12% v/v) for 1 week per concentration (MacDonnall and Marcucella, 1979; Linseman, 1987). The ethanol bottle was smaller and had a different shape than the home cage water bottle to avoid experimenter confusion. Volume of liquid consumed was measured three

times per week at the same time as food and water consumption, and in the same manner. After eliciting ethanol self-administration using the 2BC, the motivation to seek ethanol and ethanol consumption was measured in an operant LSA paradigm (Le et al., 1998; Le et al., 2000). In the operant chambers (Figure 3), number of licks from the spout of the ethanol bottle (vs. number of licks from the water bottle), and number of bar presses to receive ethanol (vs. bar presses to receive water) were measured. Two methods of ethanol self-administration were used because each method has pros and cons associated with it. Operant LSA parameters are described in detail under the "Ethanol Self-Administration" subsection under "Independent Variables" section, page 26), and using both methods maximized the amount and types of information and data that were gathered.

Open Field Activity

Open field locomotor activity is the activity of an animal when placed in a cage separate from the home cage. The activity chamber is a 40 x 40 x 30 cm clear box, with a ventilated lid, and a grid of infrared beams to measure horizontal and vertical activity. The apparatus is an AccuScan/Omnitech Electronics Digiscan system (Model RXYZCM [16 TAO], Columbus, OH; Figure 5) connected to a PC via Accuscan DCM-I-BBU analyzers (Berger, 2009; Perry, 2009; Hamilton, 2010; Long, 2010). Behaviors that were measured in the open field arena include: general movement and activity (horizontal activity and total distance traveled), exploration (vertical activity), simple learning (within session

habituation measured as area under the curve), and anxiety (time spent in the center of the apparatus). These behaviors can be altered by alcohol consumption (Hansson et al., 2006), stress (Faraday, 2002; Faraday et al., 2003; Hansson et al., 2006; Berger, 2009; Perry, 2009; Hamilton, 2010; Long, 2010), and environmental enrichment (Domjan et al., 1977; Bowling et al., 1993; Bowling and Bardo, 1994; Fernandez-Teruel et al., 1997; Faraday et al., 1999; Elliott and Grunberg, 2005; Shafer, 2005; Xu et al., 2007; Solinas et al., 2008). Animals were singly placed in 1 of 16 chambers for 1 h during the rats' active cycle (dark period). A solution of 35% isopropyl alcohol was used to clean the test arenas between subjects.

Rotarod Performance

The rotarod treadmill consists of a motor-driven drum divided into four stations (Figure 6), with constant speed or accelerating speed modes of operation. Longer latency to fall off the rotating drum indicates better balance and coordination. Rotarod performance was used to ensure that animals in the ethanol group were indeed drinking ethanol solutions (indicated by shorter latency to fall) (Newton and Ron, 2007). When a rat falls off the rotating drum (height of 26.7 cm), it breaks an infrared photobeam, stopping the timer associated with that chamber. Once all four photobeams have been broken, the drum stops rotating. Rats had three trials on this device during each day of testing; testing occurred three times throughout the experiment. Rats were measured once during the Baseline phase, once during the 2BC phase, and

once during the LSA phase. During each trial, the speed of rotation slowly increased from 0 revolutions per minute (rpm) to 35 rpm for a maximum of 3 min. The median duration on the device, expressed in seconds, was recorded for each rat on each test day. A solution of 35% isopropyl alcohol was used to clean the rotarod between subjects.

Forced Swim Test

The forced swim procedure is a rodent index of depression (Porsolt et al., 1977; Yang et al., 2006) based on the theory of learned helplessness (Seligman, 1972; Seligman and Beagley, 1975; Seligman et al., 1975; Seligman, 1978; Seligman et al., 1980) and involved training and testing on two consecutive days. On the training day, the animals were placed into cylinders of room temperature water (65 cm tall x 25 cm diameter, filled to 48 cm; Figure 7) for 15 minutes. Rats were monitored for signs of distress or difficulty swimming, but no such situation arose. At the end of the trial, animals were removed from the water, then dried and warmed using towels and heat lamps. The next day, the trial was repeated for 5 minutes under the same conditions, and the rats were tested for immobility. Total time spent immobile (*i.e.,* 75% of the animal's body is still for at least 2 seconds, as analyzed by AnyMaze [Stoelting Co., Woodale, IL] video recording software), and number of immobile episodes were measured. (Berger, 2009; Perry, 2009; Long, 2010) Testing occurred once during the 2BC phase and once during the LSA phase.

Procedure

The experiment lasted 17 weeks and was conducted in four phases: Baseline, 2-Bottle Choice (2BC) ethanol self-administration, operant liquid self-administration (LSA), and Biochemical Assays. *Post mortem* biochemical analyses occurred at the conclusion of the behavioral assessment. Two sets of rats were run in overlapping cohorts for logistical purposes in completing the behavioral testing (see Tables 1 and 2 for details). The baseline phase lasted two weeks and was necessary to acclimate the animals to daily handling by the investigators (*i.e.,* handling for 2-3 minutes for 2 days before any manipulation), to obtain pre-stress and pre-drug assessments of behaviors (*e.g.,* open field activity, rotarod), and to acclimate the animals to the 2BC paradigm, in which they had access to their normal home cage water bottle and a bottle of "0% Ethanol" (*i.e.,* "0% EtOH" or water) for 24 hours per day. Stress began on Week 2 of this phase for the animals assigned to the stress group. During this time, non-stressed animals were moved to a room separate from the housing room and stress room, so that they received a comparable environmental change and handling, but did not receive the stressor to ensure that any biological or behavioral differences found in the stressed group were the result of the stressor itself, and not the result of another variable (*e.g.,* extra handling).

The 2BC phase lasted 3 weeks. Each week, the animals in the drug condition received increasing concentrations of ethanol solution (3%, 6%, and 12% EtOH in tap water) 24 hours per day for 1 week each (MacDonnall and Marcucella, 1979; Linseman, 1987). The animals in the non-drug group received

two bottles of water for the duration of this phase. Stress continued throughout this phase as described above.

The operant self-administration phase lasted 5 weeks. The first two weeks were used to train the animals in the ethanol group to lever-press to obtain ethanol and drink from the retractable sipper tube in the chamber (32 x 13 x 12.5 cm) on a FR1 (fixed ratio 1) schedule. No water was available in the operant chamber at this time. The last three weeks of the operant phase consisted of testing in the operant chambers with increasing concentrations of ethanol (3%, 6%, and 12%) for one week each on a FR1 schedule (Le et al., 1998; Le et al., 2000). Animals in the non-drug group received water for each lever press. Stress (described above) resumed on the third week of this phase and continued for the duration of the phase.

Animals were assigned (by randomly picking rats out of shipping boxes upon arrival) to an enrichment, stress, and drug group upon arrival. Body weight, food consumption, and water consumption were measured three times per week. Locomotor activity and rotarod were measured once during each phase. Forced swim test was measured once during the 2-bottle choice phase and once during the operant phase. All measures were conducted during the dark phase (rodent active phase) of the light cycle to enhance behavioral performance and to reduce variability of biological assays. Euthanasia took place between 0900 h and 1200 h (2-5 hours after the animals' dark active cycle began). Animals in the stress group were last stressed 4-5 days before euthanasia; animals in the alcohol group had access to 12% ethanol until euthanasia. Each animal was sacrificed

by CO_2-anesthetized decapitation and trunk blood was collected for corticosterone and BAC assays. Each assay took several days to analyze all samples. Brains were removed, frozen, and later sliced and micropunched to obtain specific brain regions (VTA, NAc, and PFC) for HPLC analyses. The brain slicing and micropunching took approximately 4 weeks and HPLC analyses lasted approximately 3 weeks.

Data Analytic Strategy

Subjects were assigned (described above) to stress, housing, and drug condition. Blood-alcohol concentration (BAC) and serum corticosterone were analyzed by separate analyses of variance (ANOVAs). Parameters for the operant LSA (number of licks of ethanol, lever presses to receive ethanol, lever presses to receive water) were analyzed with univariate ANOVAs based on previous literature (*e.g.,* Le et al., 1998). Repeated-measures ANOVAs were used to analyze body weight, food consumption, water consumption, ethanol consumption (in the 2-bottle choice phase), and rotarod performance over time (for the entire experiment, and for individual phases). Open field activity was analyzed using multivariate ANOVAs (MANOVAs) because the variables of horizontal activity, vertical activity, and center time are collected together and are correlated (Long, 2010). Open field activity also was measured using repeated measures ANOVAs to assess differences within each session (to examine habituation). The forced swim test was analyzed using MANOVAs because the variables of total time spent immobile and number of immobile occurrences are

correlated. Neurotransmitter levels in the VTA, NAc, and PFC were analyzed using MANOVAs because they are correlated. Univariate ANOVAs were used to analyze individual variables for open field activity, forced swim test, and neurochemicals when significant effects were revealed with the MANOVAs. MANOVA results are reported using Pillai's trace (*V*) test statistic.

For some analyses, degrees of freedom were not consistent with N=72. There were several reasons for this occurrence. First, some data points were removed (outliers > 2 standard deviations from the mean) before statistical analysis. Also, some data were lost for two dependent variables. For open field activity during the 2BC phase, there was an equipment malfunction, and data were lost for some animals in the "No Alcohol, Stressed, Isolated" group. For the neurochemistry data, the prefrontal cortex of one animal was damaged and could not be micropunched; for several animals, the brains were sliced incorrectly and the ventral tegmental area could not be micropunched.

Several methods were implemented to reduce the probability of Type I error in the statistical analyses. The sample size (n=9 per group) for this experiment was chosen to provide sufficient power (0.80) to detect differences between and within groups (Keppel, 1991). The software nQuery Advisor (Statistical Solutions, Saugus, MA) was used to estimate the power of the independent variables and their interactions for various dependent measures that were used. These calculations were based on other research that was conducted in Dr. Grunberg's laboratory (Shafer, 2005; Berger, 2009; Perry, 2009; Starosciak and Grunberg, unpublished data). From these data, it was concluded

that the chosen sample size (n=9) would provide sufficient statistical power (on average >80%) for main effects and interactions on the dependent variables. To reduce the number of statistical tests that were performed, subsequent analyses were only completed if preliminary analyses yielded significant results (Keppel, 1991; Cohen et al., 2003). All tests were two-tailed using alpha = 0.05. Greenhouse-Geisser corrections were made (and noted in the text) on all repeated-measures ANOVAs when the assumption of sphericity was violated.

Results

All significant results ($p<0.05$, two-tailed analyses) are presented in the following text, and the relevant statistical analysis tables are located in Appendix A. All non-significant findings ($p>0.05$; two-tailed analyses) also are presented in Appendix A. In addition, figures are presented in Appendix B. Results for each of the dependent variables are presented (means ± standard error of the mean) in the following order: serum corticosterone concentration (manipulation check for the stress paradigm), body weight (check for general health), serum ethanol concentration (biological measure check of ethanol consumption), rotarod performance (behavioral manipulation check for effect of ethanol consumption), ethanol consumption in the 2BC paradigm (consummatory behavior), water consumption and food consumption (consummatory behavior check), ethanol consumption in the operant LSA paradigm (individual ethanol consumption behavior and motivation), open field activity (individual activity, exploratory

activity, anxiety index, simple learning), forced swim test (depression index), neurochemistry in the nucleus accumbens, ventral tegmental area, and prefrontal cortex. The section concludes with correlations between the neurochemical and behavioral measures.

Serum Corticosterone Concentration

Concentration of serum corticosterone was measured at the conclusion of the experiment using trunk blood collected upon euthanasia and assayed using a ^{125}I radioimmunoassay (Figure 8). As expected, stress increased levels of circulating corticosterone [547±31 vs. 448±38 ng/mL; $F(1,62)=5.609$; $p<0.05$] as compared to non-stressed animals. Stress is known to activate the hypothalamo-pituitary-adrenal (HPA) axis and increase levels of corticosterone; therefore, this measure was used as a manipulation check to ensure that lasting physiological changes occurred in response to the stressors used in this experiment. It is noteworthy that the last time the rats were stressed was approximately 4 days prior to euthanasia. Ethanol self-administration did not affect levels of serum corticosterone, indicating that alcohol consumption does not increase this index of physiological arousal. Enriched animals had greater serum corticosterone levels than did isolated animals [584±33 vs. 413 ± 31 ng/mL; $F(1,62)=17.154$; $p<0.01$]. This finding indicates that social enrichment is physiologically arousing for male rats.

Further, there was a stress x housing interaction [$F(1,62)=8.436$; $p<0.01$] such that non-stressed and stressed animals that were triple-housed (596±55

and 572±49 ng/mL, respectively) had the highest levels of corticosterone (though did not differ from each other), followed by stressed animals in the isolated condition (521±46 ng/mL), then non-stressed animals in the isolated condition (299±27 ng/mL). These data suggest that social enrichment is physiologically arousing for male rats, and that this social environment seems to attenuate the effects of predator and unpredictable stressors on serum corticosterone.

In summary, stressed (S) animals had increased serum corticosterone compared to non-stressed (NS) animals (S > NS) and socially enriched (E) animals had increased serum corticosterone compared to isolated (I) animals (E > I). Animals that consumed alcohol (A) did not differ from animals that did not consume alcohol (NA) in these levels (A=NA). There was, however, a stress x housing interaction, where enriched animals had the greatest levels of corticosterone regardless of stress condition, followed by stressed isolated animals, then non-stressed isolated animals (S E = NS E > S I > NS I).

Body Weight

Body weight was measured three times per week throughout the experiment. Because body weight data were consistent and changed gradually over the course of the experiment, three “best days” were chosen from each phase (*i.e.,* the last day of baseline, two-bottle choice, and operant liquid self-administration phases), as the data most representative of the effects of each experimental phase. Data were analyzed using a repeated-measures analysis of variance (ANOVA) with housing, stress, and alcohol conditions as the

independent variables. A Greenhouse-Geisser correction was made because the assumption of sphericity was violated. All animals gained weight throughout the course of the experiment (217±1.g during baseline, 350± 3 g during the 2BC phase, 477± 4 g during operant LSA phase) [$F(1.205,73.490)=5875.552$; $p<0.001$], indicating that animals in the stress and alcohol groups remained healthy during the experiment (Figure 9).

There was a marginally significant stress x phase interaction [$F(1.205,73.490)=3.345$; $p=0.064$], where non-stressed and stressed animals had no difference in weight during the baseline phase (this finding was expected because animals in the stress group had not yet received the stress manipulation), but had differences during the two-bottle choice phase and the operant liquid self-administration phase (both after stress manipulation had begun; Figure 10). No effects of ethanol or housing, nor interactions between these variables or interactions between these variables and phase were found. Because of the stress x phase interaction, analyses of covariance (ANCOVAs) were used to analyze body weight data within each alcohol phase, using baseline body weight as the covariate. During the 2BC phase, stress attenuated weight gain as compared to non-stressed animals [345±3 g vs. 353±4 g; $F(1,61)=7.873$; $p<0.01$] (Figure 11). This effect continued through the operant self-administration phase [471±6 g vs. 482±6 g; $F(1,61)=5.799$; $p<0.05$] (Figure 12). No effects of alcohol or housing were found in either the 2BC or operant LSA phase. No interactions occurred.

In summary, all animals gained weight over the course of the experiment (LSA > 2BC > Baseline), and stress attenuated weight gain (NS > S) during the 2BC and LSA phases.

Blood Alcohol Concentration

Trunk blood taken at the conclusion of the experiment was assayed for serum ethanol concentration using an alcohol dehydrogenase-nicotinamide adenine dinucleide (ADH-NAD) spectrophotometric reaction. As expected, rats that self-administered ethanol had greater levels of the drug in their bloodstream than did animals that did not consume ethanol [106±12 vs. 67±9 ng/dL; $F(1,61)=9.588$; $p<0.01$] (Figure 13). No significant effects were found for stress or housing conditions. There was a significant ethanol x housing interaction [$F(1,61)=8.599$; $p<0.01$], where isolated animals that self-administered ethanol (122±16 ng/dL) had the highest circulating levels of ethanol in the blood, followed by enriched animals that self-administered alcohol (90±16 ng/dL). Enriched (67±15 ng/dL) and isolated (67±10 ng/dL) rats that did not have access to alcohol had the lowest serum levels.

In summary, alcohol increased circulating levels of ethanol (A > NA) and there was an ethanol x housing interaction (A I > A E ≥ NA E = NA I).

Rotarod

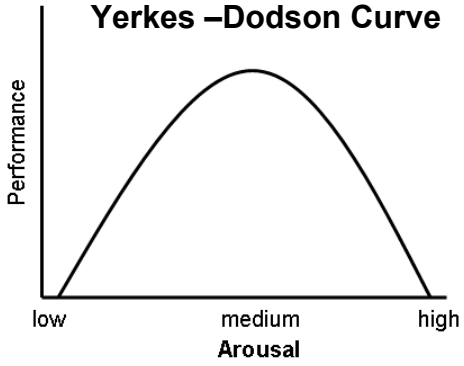
Rotarod performance was measured three times during the experiment, once during the Baseline phase, once during the 2BC phase, and once during

the operant LSA phase. Statistically significant effects and interactions are summarized below in Table 5.

Table 5 – Statistically Significant Differences for Each Treatment Group and Interactions for Rotarod

Effects and Interactions	Median Latency to Fall		
	Baseline Phase	2BC Phase	LSA Phase
Stress	No	Yes	No
Alcohol	No	Yes	No
Housing	No	No	No
Str x Alc	No	No	No
Str x Hous	No	No	No
Alc x Hous	No	No	No
Str x Alc x Hous	No	No	No

During the Baseline phase, there were no differences among groups, indicating that all groups performed equally well on this task before stress was induced or ethanol was self-administered (Figure 14). During the 2BC phase, animals that self-administered ethanol performed worse on the rotarod (*i.e.,* had shorter latencies to fall) than did animals that did not receive ethanol [52±10 vs. 83±12 s; $F(1,64)=4.205$; $p<0.05$] (Figure 15). This result was expected because ethanol is known to affect motor performance, balance, and coordination in both humans and animals (Brunton et al., 2006; Koob and Le Moal, 2006b) and was used as a manipulation check to ensure that the animals that had access to ethanol did in fact self-administer the drug. Stressed animals performed better on the rotarod (*i.e.,* had longer latencies) than did non-stressed animals [85±12 vs. 51±9 s; $F(1,64)=5.379$; $p<0.05$] (Figure 15). It is possible that this moderate stressor moved the animals along their so-called Yerkes-Dodson curves (see figure below) such that performance was improved during moderate arousal (Yerkes and Dodson, 1908). No effects of enrichment were found.



During the LSA phase, the effect of ethanol persisted, but was only marginally significant [F(1,64)=3.304; p=0.074], and the effect of stress disappeared (Figure 16). Therefore, it seems that the animals habituated to stress effects and may have become more tolerant to the effects of ethanol self-administration over time. However, all animals performed worse during this phase than during any other phase [F(1.678; 107.370)=8.816; p=0.001], and it is possible that the results were confounded by the animals' increasing size (96±9 s during baseline, 68±8 s during 2BC, 58±7 s during operant LSA). During this phase, all rats weighed 450-500 g, and many barely fit in the rotarod stations. Many of the animals resisted being placed on the treadmill, and many leapt off the treadmill within several seconds of being placed on it. It seemed the rotarod stations were somewhat uncomfortable for rats of this size, leading them to resist being placed on the rotarod, or jumping off the rotarod almost immediately.

In summary, there was no difference between groups in roatord performance during the Baseline phase (before stress or alcohol consumption occurred). During the 2BC phase, stress improved performance (S > NS), whereas alcohol consumption decreased performance (NA > A). All animals

performed poorly during the LSA phase, due to their larger size (Baseline = 2BC > LSA).

Ethanol Self-Administration in the Two-Bottle Choice

Ethanol was administered in the two-bottle choice paradigm and in the operant liquid self-administration paradigm. In the two-bottle choice paradigm, animals in the no ethanol group received an additional bottle of water in their home cages, whereas animals in the ethanol group received a bottle of ethanol solution in addition to the normal water bottle in their home cages for 24 hours per day for 4 weeks. Each week, the concentration of ethanol increased sequentially (*i.e.,* "0%" or water, 3%, 6%, 12%). Data were measured in mL per day. In this paradigm, the data were analyzed using a repeated-measures ANOVA, using the "best day" (cf. Body Weight results, pg. 46) from each concentration as the dependent variable because data were stable and to be comparable to other analyses. A Greenhouse-Geisser correction was made for degrees of freedom because the assumption of sphericity was violated. Statistically significant effects and interactions for each alcohol concentration are summarized in Table 6.

Rats drank the most ethanol or water during the 6% ethanol concentration phase (week 3 of 2BC phase; 11.7±1 mL), followed by 0% (week 1 of 2BC; 8.7±0.6 mL), then 3% (week 2 of 2BC; 8.3±0.6 mL), and 12% (week 4 of 2BC; 8.2±0.9 mL) [$F(1.822,107.481)=4.788$; $p<0.05$] (Figure 17). This finding held up regardless of whether the animals were receiving that particular concentration of

Table 6 – Statistically Significant Differences for Each Treatment Group and Interactions for Ethanol or Water Consumption in the 2BC Paradigm

Effects and Interactions	Ethanol or Water Consumption			
	0% Ethanol	3% Ethanol	6% Ethanol	12% Ethanol
Stress	No	No	No	No
Alcohol	No	No	No	No
Housing	Yes	Yes	Yes	Yes
Str x Alc	No	No	No	Yes
Str x Hous	No	No	No	Yes
Alc x Hous	No	No	No	No
Str x Alc x Hous	No	No	No	No

ethanol or water during the whole paradigm. There was no difference between non-stressed and stressed rats nor a difference between alcohol and no alcohol groups for amount of liquid consumed from the additional bottle of fluid, meaning non-stressed and stressed rats consumed the same amount of fluid, and animals in the no alcohol group drank the same volume of water as animals in the alcohol group drank of alcohol. Enriched animals drank more than isolated animals [12.1±0.8 vs. 6.2±0.6 mL; $F(1,59)=31.684$; $p<0.001$] regardless of the fluid (water or alcohol) they were consuming. There also was a housing x phase interaction [$F(1.822,107.481]=7.054$; $p<0.01$] where overall, enriched animals drank more than isolated animals, but enriched animals drank more during the 6% ethanol phase and less during the 12% ethanol phase. Conversely, isolated animals drank most during the 0% ethanol phase and less during the 6% ethanol phase.

Each ethanol concentration phase was then analyzed using an ANCOVA with "0% ethanol" (*i.e.,* water) consumption (Figure 18) as the covariate. No effect of stress or ethanol was found, meaning that all animals drank the same amount of fluid available from the extra water bottle regardless of whether they were stressed or non-stressed, or whether they had access to water or alcohol

solution. During the 3% [10.9±0.7 mL; $F(1,60)=4.893$; $p<0.05$] (Figure 19), 6% [16.7±1.6 vs. 6.4±1.0 mL; $F(1,60)=12.229$; $p=0.001$] (Figure 20), and 12% [10.1±1.3 vs. 6.2±1.0 mL; $F(1,60)=4.325$; $p<0.05$] (Figure 21) ethanol phases, enriched animals drank more than isolated animals. Several two-way interactions were found during the 12% ethanol phase. There was a stress x ethanol interaction [$F(1,60)=3.965$; $p=0.051$] where non-stressed animals drank more ethanol than did stressed animals (11.6±2.7 vs. 6.0±0.1 mL), but stress did not affect animals in the no alcohol group (7.1±1.2 mL for non-stressed vs. 7.9±1.5 mL for stressed). A stress x housing interaction [$F(1,60)=8.872$, $p<0.01$] also was found in which non-stressed enriched animals drank the most (12.2±2.4 mL) , whereas non-stressed isolated animals drank the least (5.5±1.3 mL).

In summary, alcohol had no effect on amount of liquid consumed in this paradigm, meaning animals in the no alcohol group drank equal amounts of water as animals in the alcohol group drank of alcohol (NA = A). Further, rats in the socially enriched housing condition consumed more liquid that rats in the isolated housing condition (E > I). There was a stress x alcohol interaction (NS A > S A > NS NA = S NA) and a stress x housing interaction (NS E > S E = S I > NS I) during the week that rats in the alcohol group received 12% ethanol.

Water Consumption

Water consumption was measured three times per week throughout the duration of the experiment. Data were recorded as milliliters of water consumed per day. The data were analyzed using a repeated-measures ANOVA, using the

“best day” (cf. Body Weight results, pg. 46) from each phase (Baseline, 2BC, LSA) as the dependent variables because data were stable and to be comparable to other analyses. A Greenhouse-Geisser adjustment was made on the degrees of freedom because the assumption of sphericity was violated.

All animals drank more water over time (29.3± 1.1 mL during baseline, 40.1± 1.6 mL during 2BC, 42.6± 1.2 mL during LSA) [$F(1.685,97.723)=36.329$; $p<0.001$] indicating that the rats remained healthy during the experiment (Figure 22). Time interacted with each independent variable. Stressed animals drank more water during the operant LSA phase than during the 2BC phase, but this effect was reversed for non-stressed animals [$F(1.685,97.723)=4.998$; $p<0.05$]. Both stressed and non-stressed animals drank less water during the baseline phase. Animals that did not receive ethanol consumed more water during the operant LSA phase than the 2BC phase [$F(1.685,97.723)=3.733$; $p<0.05$]. This effect was reversed for animals that did receive ethanol. Animals in both groups drank less water during the baseline phase. Enriched animals drank more water during the 2BC phase than in the operant LSA phase [$F(1.685,97.723)=4.995$; $p<0.05$]. This pattern was reversed for isolated animals. Water consumption was less during the baseline phase for both enriched and isolated animals. Further, enriched animals drank more water than isolated animals [$F(1,58)=19.633$; $p<0.001$] overall. A stress x housing interaction [$F(1,58)=12.395$; $p=0.001$] was found such that non-stressed animals in the enriched condition had greater water consumption than stressed animals in this condition. Stressed animals drank less water regardless of housing condition.

Though there were no differences between groups at baseline (Figure 23), ANCOVAs were run for each phase, using baseline water consumption as a covariate. During the two-bottle choice phase (Figure 24), there was a main effect of stress [$F(1,60)=9.686$; $p<0.01$] and a main effect of housing [$F(1,60)=18.446$; $p<0.001$]. Alcohol group did not affect water consumption. Non-stressed animals drank more water than stressed animals (44.1±2.8 vs. 36.0±1.2 mL), and enriched animals drank more water than isolated animals (45.3±2.3 vs. 35.5±1.8 mL). There also was a stress x housing interaction [$F(1,60)=7.249$; $p<0.01$]; this pattern was similar to the one described in the previous paragraph. In the liquid self-administration phase (Figure 25), there was a main effect of housing [45.2±1.3 vs. 40.0±2.0 mL; $F(1,61)=5.397$; $p<0.05$] and a stress x housing interaction [$F(1,61)=7.597$; $p<0.01$]; again, these patterns were similar to the ones previously described.

In summary, all rats continued to drink more water over the course of the experiment (LSA > 2BC > Baseline). Non-stressed animals consumed more water than did stressed animals during the 2BC phase (NS > S); enriched animals drank more water than did isolated animals (E > I) during the 2BC phase and operant LSA phase.

Food Consumption

Food consumption was measured three times a week for the duration of the experiment and is reported as grams consumed per day. The data were analyzed using a repeated-measures ANOVA, using the “best day” (cf. Body

Weight results, pg. 46) from each phase (Baseline, 2BC, LSA) as the dependent variables because data were stable and to be comparable to other analyses. Using a repeated-measures ANOVA, animals consumed the most food during the two-bottle choice phase, followed by (29.0±0.4 g) the operant self-administration phase (28.4±0.4 g), and then by the baseline phase (24.2± 0.4 g) [$F(2,122)=71.967$; $p<0.001$] (Figure 26). There was an ethanol x phase interaction [$F(2,122)=3.800$; $p<0.05$], such that animals receiving ethanol consumed more food than animals not receiving ethanol during the two-bottle choice phase and the baseline phase (though no animal received ethanol during the baseline phase); during the operant liquid self-administration phase, both groups consumed similar amounts of food. During the baseline phase (Figure 27), there were no main effects of stress or ethanol group, but socially enriched animals consumed more food than isolated animals [24.6±0.6 vs. 23.8±0.4 g; $F(1,61)=4.473$; $p<0.05$]. There was a stress x housing interaction [$F(1,61)=8.533$; $p<0.01$], where non-stressed animals in the enriched condition had the greatest food consumption (25.0±1.1 g), followed by stressed isolated animals (24.3±0.7 g) and stressed enriched animals (24.2±0.2 g). Non-stressed animals in the isolated condition consumed the least amount of food (23.3±0.4 g). Because of this difference at baseline, the 2BC and operant LSA phases were individually analyzed using analyses of covariance (ANCOVAs), with baseline food consumption as the covariate. These effects are described in the following paragraph.

Using an ANCOVA with baseline food consumption as a covariate, a stress x housing interaction [$F(1,61)=4.578$; $p<0.05$] was found for the 2BC phase (Figure 28) similar to the interaction at baseline as described above, but no other effects or interactions emerged. During the operant LSA phase, stressed animals consumed more food than non-stressed animals [29.1±0.3 vs. 27.8±0.7 g; $F(1,62)=3.896$; $p=0.053$] (Figure 29); no effects of ethanol or housing were found. There was a stress x housing interaction [$F(1,62)=5.533$; $p<0.05$] in this phase as well, though the pattern was slightly different. Stressed animals in the isolated condition had the greatest food consumption (29.8±0.6 g), followed by non-stressed enriched (28.6±0.7 g), stressed enriched (28.3±0.2 g), and finally, non-stressed isolated (26.9±1.2 g). An ethanol x housing interaction [$F(1,62)=4.295$; $p<0.05$] was found, where enriched animals not receiving ethanol consumed the most food (29.3±0.3 g), followed by isolated animals receiving ethanol (29.0±0.6 g), and enriched animals receiving ethanol (28.8±1.3 g). Isolated animals not receiving ethanol consumed the least amount of food (27.6±0.6 g). Finally, there was a 3-way stress x ethanol x housing interaction [$F(1,62)=4.038$; $p<0.05$]. The greatest difference in food consumption was seen between isolated stress groups not receiving ethanol (*i.e.,* stressed > non-stressed). This effect was reversed in enriched animals not receiving ethanol. Ethanol consumption attenuated effects of stress and housing.

In summary, rats consumed the most food during the 2BC phase (2BC > LSA > Baseline). At baseline, socially enriched animals ate more food than did isolated animals (E > I) and there was a stress x housing interaction (NS E > S I

= S E > NS I), even though stress had not yet begun. For this reason, baseline food consumption was covaried for the 2BC and operant LSA phases. During the 2BC phase, the same stress x housing interaction continued (NS E > S I = S E > NS I), but after stress had begun. During the operant LSA phase, stressed animals consumed more food than did non-stressed animals (S > NS) and there was an alcohol x housing interaction (NA E > A I > A E > NA I). The stress x housing interaction continued in this phase, but with a slightly different pattern than during the Baseline and 2BC phases (S I > NS E > S E > NS I).

Ethanol Consumption in Operant Liquid Self-Administration

In the operant liquid self-administration paradigm, only animals in the alcohol group were tested. Animals in the no alcohol group spent the same amount of time in the apparatus but were not exposed to the self-administration paradigm. Data for number of licks on the ethanol sipper tube, number of lever presses to receive ethanol, and number of lever presses to receive water were averaged over the course of the week for each concentration phase. ANOVAs were used to analyze each measure during each concentration phase. Number of lever presses for water was considered within each ethanol concentration phase to control for amount of activity in the operant chambers. Stress and housing did not affect number of lever presses for water at any concentration. These results are summarized in Table 7.

Table 7 – Statistically Significant Differences among Groups and Interactions for Variables in the Operant LSA Paradigm

Effects and Interactions	3% Ethanol			6% Ethanol			12% Ethanol		
	# Licks (Eth)	# Bar Press (Eth)	# Bar Press (H_2O)	# Licks (Eth)	# Bar Press (Eth)	# Bar Press (H_2O)	# Licks (Eth)	# Bar Press (Eth)	# Bar Press (H_2O)
Stress	No	No	No	No	No	No	No	No	No
Housing	Yes	Yes	No	No	Yes	No	No	Yes	No
Str x Hous	No	No	No	No	No	No	No	Yes	No

Key:
Licks (Eth) = Number of licks from ethanol sipper tube
Bar Press (Eth) = Number of bar presses to receive ethanol
Bar Press (H_2O) = Number of bar presses to receive water

During the week of 3% ethanol in the operant LSA phase, isolated animals took more licks [27.3±3.0 vs.10.8±1.4; $F(1,32)=7.733$; $p<0.01$] from the ethanol sipper tube (Figure 30) and pressed the ethanol lever more times [3.6±0.5 vs.1.8±0.2; $F(1,32)=4.595$; $p<0.05$] (Figure 31) than did animals in the enriched condition. When the animals received 6% ethanol, isolated animals lever pressed to receive ethanol more than enriched animals [1.8±0.2 vs. 1.0±0.1; $F(1,32)=4.739$; $p<0.05$] (Figure 34). No effect of stress or housing x stress interactions were found for the weeks of 3% or 6% ethanol. During the week of 12% ethanol, isolated animals lever-pressed to receive ethanol more times [2.3±0.3 vs. 1.0±0.2; $F(1,32)=9.731$; $p<0.01$] (Figure 36) than did animals in the enriched condition. Further, there was a housing x stress interaction [$F(1,32)=5.074$; $p<0.05$] where isolated animals that were stressed had the greatest number of lever-presses for ethanol (2.9±0.5). Stressed animals in the enriched condition (1.0±0.2), and non-stressed animals in isolated (1.5±0.2) and enriched (1.1±0.2) conditions lever-pressed approximately the same amount to obtain ethanol.

In summary, it seems that animals housed alone consume more alcohol in the operant paradigm than do animals housed in groups of three (I > E). Because of the possibility that isolated animals were more active in the operant chambers than enriched animals, lever pressing for water was taken into consideration. Neither housing condition nor stress condition had a statistically significant effect on lever pressing for water. Therefore, one can conclude that the isolated rats were more motivated to consume alcohol than socially enriched animals, and were not just more active.

Open Field Locomotor Activity

Open field locomotor activity was measured once each during the baseline, 2BC, and operant LSA phase and data for the variables of horizontal activity, total distance traveled, vertical activity, and center time were collected. Data were analyzed collectively for each session using a MANOVA. Each variable also was analyzed by univariate ANOVAs during each phase; the results were consistent with the MANOVA findings and are not presented in the text. Statistical tables for both types of analyses are located in Appendix A. During the baseline phase, the MANOVA yielded a statistically significant effect of housing [V=0.433; $F(4,61)=11.628$; $p<0.001$] and a stress x housing interaction [V=0.150; $F(4,61)=2.686$; $p<0.05$]. During the 2BC phase, the MANOVA yielded a main effect of housing [V=0.270; $F(4,53)=4.910$; $p<0.01$]. A main effect of housing also was found during the operant LSA phase [V=0.284; $F(4,61)=6.046$;

p<0.001]. A summary of MANOVA and ANOVA significant effects for open field data is presented below in Table 8.

Table 8 – Significant Differences for Each Treatment Group and Interactions for Locomotor Horizontal Activity, Total Distance Traveled, Vertical Activity, and Center Time

Effects and Interactions	Baseline Phase				2BC Phase				LSA Phase			
	HA	TD	VA	CT	HA	TD	VA	CT	HA	TD	VA	CT
Stress	No	No	No	No	No	No	No	No	No	No	No	No
Alcohol	No	No	No	No	No	No	No	No	No	No	No	No
Housing	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes
Str x Alc	No	No	No	No	No	No	No	No	No	No	No	No
Str x Hous	Yes	No	No	No	Yes	Yes	No	No	No	No	Yes	No
Alc x Hous	No	No	No	No	No	No	No	No	No	No	No	No
Str x Alc x Hous	No	No	No	No	No	No	No	No	No	No	No	No

Key: HA = Horizontal Activity TD = Total Distance Traveled
VA = Vertical Activity CT = Center Time

Horizontal Activity and Total Distance Traveled

Horizontal activity and total distance traveled in an open field locomotor apparatus are indicative of general animal health as well as an index of overall activity. No effect of stress or ethanol consumption was found for either of these parameters during the baseline phase, but isolated animals had greater horizontal activity [19460±525 vs. 14525±555 beam breaks; F(1,64)=44.596; p<0.001] (Figure 39) and traveled further [8678±319 vs. 5961±259 cm; F(1,64)=43.170; p<0.001] (Figure 42) than did animals in the social enrichment condition. There was a stress x housing interaction for horizontal activity [F(1,64)=6.270; p<0.05] (Figure 39) in which isolated animals subjected to stress had the greatest activity (20215±696 beam breaks), whereas enriched animals in the stressed condition had the least activity (13430±772 beam breaks). These

findings suggest that the housing effect is almost immediate (less than two weeks in the housing condition). The socially enriched animals were removed from their normal groups of three and placed individually into the open field chambers. Even though this was a novel environment, there was less overall stimulation than in the normal housing condition, leading to reduced activity. In contrast, rats that were individually housed had more sensory stimulation in the novel open field environment, thereby leading them to be more active. Further, there appeared to be a difference in activity between the stress and no stress conditions (because stress interacted with housing), even though stress had not yet been induced.

During the 2BC phase, there was no effect of stress or alcohol, but the effect of housing persisted, where isolated animals exhibited greater horizontal activity [21446±1206 vs. 16500±769 beam breaks; $F(1,56)=17.17.261$; $p<0.001$] (Figure 40) and traveled a further distance [11310±742 vs. 8121±409 cm; $F(1,56)=20.323$; $p<0.001$] (Figure 43) than did enriched animals. Again, the effect of differences in sensory stimulation in the home cage versus the open field chamber is apparent for isolated and enriched rats. There was a stress x housing interaction for horizontal activity [$F(1,56)=5.017$; $p<0.05$] (Figure 40) and total distance traveled [$F(1,56)=4.184$; $p<0.05$] (Figure 43), where isolated animals that were stressed had the greatest activity (24097±2653 beam breaks; 12803±1544 cm), but enriched animals that were stressed had the least activity (15647±756 beam breaks; 7725±504 cm) for each of these parameters. It

appears that stress increases activity for isolated animals, but reduces activity in socially enriched animals.

Similar to the 2BC phase, there were no effects of stress or ethanol in the operant LSA phase, but the housing effect continued. Isolated animals had greater horizontal activity [17499±485 vs. 14867±607 beam breaks; $F(1,64)=11.393$; $p=0.001$] (Figure 41) and traveled further [9465±334 vs. 7578±370 cm; $F(1,64)=14.238$; $p<0.001$] (Figure 44) than did enriched animals. In this phase, stress appeared to attenuate housing differences, in contrast to the 2BC phase, where stress seemed to enhance housing differences.

In summary, rats in the isolated condition had greater horizontal activity and traveled a further distance in the open field apparatus than did socially enriched rats (I > E). A stress x housing interaction (S I > NS I = NS E > S E) occurred during baseline (even though stress had not yet begun) and during the 2BC phase.

Vertical Activity

Vertical activity, or rearing on the hind legs, is often used as an index of exploratory activity. There was no effect of stress or ethanol in any phase, but rats in the isolated condition had more vertical activity during the baseline [1465±60 vs. 1120±74 beam breaks; $F(1,64)=13.213$; $p=0.001$] (Figure 45), 2BC [2663±149 vs. 2145±99 beam breaks; $F(1,56)=10.483$; $p<0.01$] (Figure 46), and operant LSA [3151±131 vs. 2571±116 beam breaks; $F(1,64)=11.656$; $p=0.001$] (Figure 47) phases than did enriched animals. A stress x housing interaction

was found for vertical activity [$F(1,64)=6.182$; $p<0.05$] (Figure 47) in which non-stressed isolated rats showed the most exploratory behavior (3467±158 beam breaks) and the non-stressed enriched rats exhibited the least exploratory behavior (2466±150 beam breaks) during the operant LSA phase. Stress appeared to attenuate housing effects during this phase.

In summary, as with horizontal activity and total distance traveled, social enrichment decreased vertical activity in the open field apparatus (I > E). During the operant LSA phase, there was a stress x housing interaction (NS I > S I = S E > NS E) for vertical activity where social enrichment attenuated the effects of stress.

Center Time

Time spent in the center of the open field apparatus has been used to index anxiety, with more time spent in the center indicating less anxiety. Similar to the other open field variables, there were no individual effects of stress or ethanol consumption, though isolated rats spent more time in the center of the apparatus during the baseline [605±38 vs. 465±30 s; $F(1,64)=8.553$; $p<0.01$] (Figure 48) and operant LSA [1683±71 vs. 1405±79 s; $F(1,64)=6.688$; $p<0.05$] (Figure 50) phases than did enriched animals. There were no main effects or interactions for center time during the 2BC phase (Figure 49).

In summary, it appears that ethanol consumption does not reduce anxiety-like behaviors in this paradigm, nor does stress increase anxiety-like behavior.

Habituation

Within-session habituation to the open field apparatus is the gradual reduction in horizontal activity over the course of the 1 hour testing period and is thought to signify simple learning (Paulus et al., 1998; Varty et al., 2000; Elliott and Grunberg, 2005). Faster habituation to the open field apparatus is indicative of better learning capacity. Habituation data were collected in 5 minute bins over the course of the hour test period (12 bins total). Data were analyzed using a repeated-measures ANOVA within each session; Greenhouse-Geisser adjustments for degrees of freedom were made when the assumption of sphericity was violated.

During the baseline phase (Figure 51), activity for all rats decreased during the test session [$F(7.829,501.077)=129.629$; $p<0.001$]. There also was a housing x time interaction [$F(7.829,501.077)=2.660$;$p<0.01$], such that socially enriched animals habituated faster to the open field apparatus faster than animals housed in isolation.

Activity also decreased for all animals in the 2BC phase [$F(7.584,424.724)=147.608$;$p<0.001$], and there was a stress x alcohol x time interaction [$F(7.584,424.724)=2.517$; $p<0.05$] (Figure 52) such that stressed rats that did not consume alcohol habituated to the open field environment the fastest, and stressed rats that did consume alcohol habituated the slowest. Non-stressed rats had intermediate rates of habituation.

All rats habituated to the open field environment during the operant LSA phase [$F(11,704)=262.762$; $p<0.001$] (Figure 53). There was a housing x time

interaction [$F(11,704)=2.041$; $p<0.05$] in which enriched animals habituated more quickly than isolated animals. Further, there were stress x alcohol x time [$F(11,704)=2.585$; $p<0.01$] and stress x housing x time [$F(11,704)=2.570$; $p<0.01$] interactions. With regard to the first interaction, the pattern was much more complex than the one described for the 2BC phase – essentially, habituation in the beginning of the session was different than the end of the session depending on stress and alcohol group. With regard to the second interaction, the pattern was clearer. Non-stressed enriched animals habituated faster than stressed enriched, but there was no difference in habituation among stress groups in the isolated condition.

In summary, all rats habituated to the open field apparatus in each phase, but socially enriched animals habituated faster. Further, there were interactions between habituation, stress, alcohol, and housing conditions, leading to complex patterns. The clearest finding is that enrichment causes increased habituation to the open field environment, suggesting that simple learning is enhanced in this group of animals.

Forced Swim Test

Total time spent immobile and number of immobile episodes during the 5-minute forced swim test was measured once during the 2BC phase and once during the LSA phase. In this paradigm, more immobility (as measured by these two variables) indicates a greater depressive index in rats (a model of “learned helplessness,” or giving up trying to escape), or simply, more depression

(Seligman, 1972; Seligman and Beagley, 1975; Seligman et al., 1975; Porsolt et al., 1977; Seligman, 1978; Seligman et al., 1980). A MANOVA was used to analyze variables during each phase, and univariate ANOVAs analyzed each variable separately within each phase. Because the results from both analyses were similar, only the MANOVA results are presented in the text. Tables for both types of statistical analyses are located in Appendix A. Although the overall MANOVAs only yielded marginally significant results, but individual, exploratory ANOVAs show that there may have been some interesting findings. The significant results are summarized below in Table 9.

Table 9 – Significant Differences for Each Treatment Group and Interactions for Forced Swim Test Measures

Effects and Interactions	2BC Phase		LSA Phase	
	Time Spent Immobile	# Immobile Episodes	Time Spent Immobile	# Immobile Episodes
Stress	No	No	No	No
Alcohol	No	No	No	No
Housing	No	No	Yes	Yes
Str x Alc	Yes	Yes	Yes	Yes
Str x Hous	No	No	No	No
Alc x Hous	Yes	No	No	No
Alc x Hous x Str	No	No	Yes	No

During the 2BC phase, no individual effects of any independent variable were found. There was a stress x ethanol interaction for total time spent immobile [$F(1,63)=8.473$; $p<0.01$] (Figure 54) and number of immobile episodes [$F(1,63)=10.177$; $p<0.001$] (Figure 55) where non-stressed animals that self-administered ethanol exhibited the greatest depression (32.3±7.1 s; 12.3±2.4), whereas non-stressed animals that did not consume ethanol had the lowest

depression (14.2±2.6 s; 5.8±0.9). Stressed animals had intermediate depressive indices, and ethanol self-administration seemed to reduce depression in these animals. There was an ethanol x housing interaction for total time spent immobile [$F(1,63)=4.172$; $p<0.05$] (Figure 54) such that isolated animals that received ethanol showed the greatest depressive behavior (29.3±7.1 s), whereas isolated animals that did not self-administer ethanol exhibited the least depressive behavior (16.3±3.1 s). Enriched animals showed intermediate depressive behavior, and ethanol self-administration reduced depressive behavior in enriched rats. From these data, it appears that ethanol acted as an anti-depressant for stressed animals during this phase. It is possible that the rats with access to alcohol self-administered the drug as self-medication to reduce possible stress-induced depression.

In the operant LSA phase, no effects of stress or alcohol were apparent, but enriched animals showed greater depressive behavior for total time spent immobile [36.7±4.5 vs. 25.8±2.9 s; $F(1,61)=5.471$; $p<0.05$] (Figure 56) and number of immobile episodes [14.3±1.6 vs. 10.7±1.3; $F(1,61)=7.513$; $p<0.01$] (Figure 57) than isolated animals. A stress x ethanol interaction for total time spent immobile [$F(1,61)=8.550$; $p<0.01$] (Figure 56) and number of immobile episodes [$F(1,61)=6.878$; $p<0.05$] (Figure 57) was found, such that non-stressed animals that consumed ethanol had the greatest depressive index (39.5±6.1 s; 15.6±2.4), but stressed animals that self-administered ethanol had the lowest depressive index (20.1±3.1 s; 9.3±1.6). Animals that did not self-administer ethanol showed intermediate depressive behavior, with stress increasing

depression in these animals. Further, there was a complex ethanol x housing x stress interaction for total time spent immobile [F(1,61)=3.984; p=0.05] (Figure 56). In animals that did not consume ethanol, stress increased depressive behavior in enriched animals only, and had no effect in isolated animals. In animals that did self-administer ethanol, depressive behavior was reduced overall, but especially in stressed animals in both housing conditions.

In summary, it appears that enrichment increased depressive behavior, especially for animals in the stressed condition. It is possible that being removed from cage mates and placed in the swim apparatus was a greater stressor for the enriched animals than for the isolated animals, thereby making them more likely to exhibit depressive behavior. Also, it seems that ethanol acted as a depressant in non-stressed animals (reasonable because it is a central nervous system depressant drug), but as an anti-depressant in stressed animals. Just as in the 2BC phase, it seems that the rats (in the alcohol group) may have self-administered ethanol as a way to self-medicate or reduce stress-induced depression.

Neurochemicals

There were significant effects among neurochemicals (DA, DOPAC, HVA, NE, 5-HT, and 5-HIAA) depending on the brain region (VTA, NAc, and PFC); the findings are summarized in Table 10. The significant results are discussed in detail below. Significant findings for NAc, VTA, and PFC are summarized in Tables 11, 12, and 13, respectively.

Table 10 – Significant Differences among any Treatment Groups for Neurochemicals in Each Brain Region

Brain Region	Neurochemical					
	DA	DOPAC	HVA	NE	5-HT	5-HIAA
NAC	Yes	No	Yes	Yes	No	Yes
VTA	Yes	No	Yes	Yes	No	No
PFC	No	Yes	Yes	No	Yes	No

Neurochemical data are presented by brain region starting with the nucleus accumbens, followed by the ventral tegmental area (the two main brain regions associated with reward and reinforcement), then the prefrontal cortex (associated with executive functioning and inhibitory control over reinforcement). Data within each brain region are presented according to statistically significant effects of independent variables (starting with stress, followed by alcohol and housing, then interactions) on each neurochemical starting with dopamine (the neurochemical most often associated with reward and reinforcement), followed by its metabolites (DOPAC and HVA). Next, norepinephrine is discussed because it too is classified as a catecholamine and its precursor is dopamine. Serotonin and its metabolite, 5-HIAA, are presented last because they are in a different class of chemicals (indoleamines), are less important in the reward pathway *per se*, but are key neurochemicals in the modulation of mood. Data from MANOVAs are discussed first, followed by ANOVA findings. All supporting statistical tables appear in Appendix A.

Nucleus Accumbens

The overall MANOVA for neurochemicals in the NAc yielded a main effect of stress [*V*=0.245; F(6,51)=2.762; p<0.05] and a main effect of housing [*V*=0.611; F(6,51)=13.378; p<0.001]. The table below summarizes the significant findings.

Table 11 - Significant Differences for Each Treatment Group and Interactions for Neurochemicals in the Nucleus Accumbens

Effects and Interactions	Neurochemical					
	DA	DOPAC	HVA	NE	5-HT	5-HIAA
Stress	No	No	No	Yes	No	Yes
Alcohol	No	No	No	No	No	Yes
Housing	Yes	No	Yes	Yes	No	Yes
Str x Alc	No	No	No	No	No	No
Str x Hous	No	No	No	Yes	No	No
Alc x Hous	No	No	No	Yes	No	No
Str x Alc x Hous	No	No	No	No	No	No

In the nucleus accumbens, non-stressed animals had greater levels of NE [13.7±0.8 vs. 11.4±0.5 pg/µg; F(1,56)=8.525; p<0.01] (Figure 61) and 5-HIAA [7.1±0.3 vs. 6.0±0.4 pg/µg; F(1,56)=8.603; p<0.01] (Figure 63) than did stressed animals. The effect of stress was not significant for DA, DOPAC, HVA, and 5-HT. Animals that did not self-administer ethanol had higher levels of 5-HIAA than did animals that self-administered ethanol [6.8±0.4 vs. 6.4±0.4 pg/µg; F(1,56)=4.889; p<0.05] (Figure 63); but no significant effect of ethanol was found for any other neurochemical. Social enrichment increased DA [156.5±9.5 vs. 138.1±9.8 pg/µg; F(1,56)=5.129; p<0.05] (Figure 58), HVA [14.8±0.7 vs. 13.3± 0.7 pg/µg; F(1,56)=4.786; p<0.05] (Figure 60), and NE [13.5±0.8 vs. 11.6±0.5 pg/µg; F(1,56)=5.849; p<0.05] (Figure 61), but decreased 5-HIAA [5.3±0.2 vs. 7.8±0.4 pg/µg; F(1,56)=37.753; p<0.001] (Figure 63) as compared to isolated

individuals. Effects of housing condition on DOPAC and 5-HT were not significant.

Several significant interactions also were found in the nucleus accumbens. There was a stress x housing interaction on HVA [$F(1,56)=4.855$; $p<0.05$] and NE [$F(1,56)=7.503$; $p<0.01$]. Enriched animals that were non-stressed had the greatest levels of HVA (16.7±1.2 pg/µg), whereas non-stressed isolated animals had the lowest levels of HVA (12.9±1.0 pg/µg; Figure 60). Conversely, for norepinephrine, non-stressed enriched animals had the highest levels of NE (16.0±1.2 pg/µg) in the nucleus accumbens, and stressed enriched animals had the lowest levels of NE (11.2±0.6 pg/µg; Figure 61). In addition, there was an ethanol x housing interaction for norepinephrine [$F(1,56)=4.011$; $p=0.05$] (Figure 61), where socially enriched animals that self-administered ethanol had the highest levels of NE (14.6±1.4 pg/µg), whereas isolated animals that self-administered ethanol had the lowest levels of NE (11.0±0.6 pg/µg). This interaction was non-significant for the other five neurochemicals.

In the NAc, several neurochemical findings disappear (*i.e.,* become not statistically significant) when the univariate ANOVA was used, while other results remain statistically significant. For DA and HVA, the effect of housing disappears, but the stress x housing interaction for HVA remains. All effects and interactions remain significant for NE. For 5-HIAA, the effect of alcohol self-administration disappeared, but the stress x housing interaction remained significant.

In summary, stress decreased NE and the serotonin metabolite 5-HIAA (NS > S) in the NAc. Alcohol consumption decreased 5-HIAA (NA > A). The most consistent finding in this brain region was the effect of housing condition. Social enrichment increased dopamine, HVA, and NE (E > I), but decreased 5-HIAA (I > E). It appears that different housing conditions may differentially affect catecholamines and indoleamines. Along with these main effects, there was a stress x housing interaction for HVA (NS E > S I = S E > NS I) and NE (NS E > NS I > S I > S E). NE also had an alcohol x housing interaction (A E > NA E > NA I > A I). It is interesting and important to point out that for these neurochemistry data, statistically significant findings can change depending on the analysis used (this finding is consistent for the next two brain regions as well).

Ventral Tegmental Area

The MANOVA for neurochemicals in the VTA only produced a significant three-way stress x alcohol x housing interaction [V=0.320; $F(6,41)=3.213$; $p<0.05$], but the between-subjects effects and individual ANOVAs yielded additional results, which are summarized in the table below and discussed in the text.

Table 12 – Significant Differences for Each Treatment Group and Interactions for Neurochemicals in the Ventral Tegmental Area

Effects and Interactions	Neurochemical					
	DA	DOPAC	HVA	NE	5-HT	5-HIAA
Stress	No	No	No	No	No	No
Alcohol	Yes	No	Yes	Yes	No	No
Housing	No	No	No	No	No	No
Str x Alc	No	No	No	No	No	No
Str x Hous	No	No	No	No	No	No
Alc x Hous	No	No	No	No	No	No
Str x Alc x Hous	No	No	No	Yes	No	No

In the ventral tegmental area, there were no effects of stress on any of the six neurochemicals, but there was an effect of alcohol consumption. Animals with access to ethanol had greater levels of DA [44.5±1.8 vs. 38.1±1.8 pg/µg; $F(1,53)=6.559$; $p<0.05$] (Figure 64), HVA [7.6±0.2 vs. 6.8±0.3 pg/µg; $F(1,52)=4.498$; $p<0.05$] (Figure 66), and NE [18.4±0.5 vs. 16.8±0.6 pg/µg; $F(1,51)=4.484$; $p<0.05$] (Figure 67) than did rats without access to ethanol. No effect of ethanol was found for DOPAC (Figure 65), 5-HT (Figure 68), or 5-HIAA (Figure 69). There were no differences between isolated and enriched animals for any of the six neurochemicals examined. Two-way interactions between ethanol, housing, or stress groups for any of the six neurochemicals in the ventral tegmental area were not found, but there was a three-way stress x ethanol x housing interaction for NE [$F(1,51)=4.004$; $p=0.05$] (Figure 67), as described in the following sentences. In the non-ethanol group, stress increased the amount of norepinephrine in the VTA for isolated animals, but had no effect on enriched animals. In animals that received ethanol, ethanol increased the amount of norepinephrine for both housing groups and stress groups, but attenuated the stress effect in isolated animals only.

When univariate ANOVAs were used to analyze individual neurochemicals, the effect of alcohol on DA was lost, but a stress x housing interaction emerged [$F(1,62)=5.063$; $p<0.05$] that was not apparent in the MANOVA. Effects of alcohol on HVA and NE remained, and the 3-way interaction for NE remained.

In summary, alcohol increased DA, HVA, and NE (A > NA) in the VTA. There was a three-way stress x alcohol x housing interaction for NE. Again, differences were observed between MANOVA and individual ANOVA significant findings.

Prefrontal Cortex

The MANOVA only yielded a statistically significant main effect of alcohol [$V=0.304$; $F(6,42)=3.063$; $p<0.05$], although follow-up ANOVAs and individual ANOVAs do not show any effects of alcohol. This finding may mean that there was an overall effect of alcohol to subtly alter the six neurochemical levels, but was not statistically significant for any individual neurochemical. The results of follow-up and individual ANOVAs are summarized in the table below.

Table 13 - Significant Differences for Each Treatment Group and Interactions for Neurochemicals in the Prefrontal Cortex

Effects and Interactions	Neurochemical					
	DA	DOPAC	HVA	NE	5-HT	5-HIAA
Stress	No	No	No	No	No	No
Alcohol	No	No	No	No	No	No
Housing	No	No	No	No	Yes	No
Str x Alc	No	No	No	No	No	No
Str x Hous	No	No	No	No	No	No
Alc x Hous	No	Yes	Yes	No	Yes	No
Alc x Hous x Str	No	No	No	No	No	No

In the prefrontal cortex, neither stress nor alcohol had any effect on the six assayed neurochemicals; however, isolated animals had greater levels of 5-HT [7.4±0.1 vs. 2.1±0.2 pg/µg; $F(1,47)=5.154$; $p<0.05$] than socially enriched animals (Figure 74). There were no other housing effects on the other neurochemicals in this brain region.

Only one interaction was found for this brain region, an ethanol x housing interaction for DOPAC [$F(1,47)=4.149$; $p<0.05$], HVA [$F(1,47)=7.270$; $p<0.01$], and 5-HT [$F(1,47)=8.044$; $p<0.01$]. For DOPAC (Figure 71) and HVA (Figure 72), enriched animals receiving ethanol (DOPAC: 1.9±0.1 pg/µg; HVA: 2.8±0.2 pg/µg), isolated animals not receiving ethanol (DOPAC: 1.9±0.1 pg/µg; HVA: 2.7±0.2 pg/µg), and isolated animals receiving ethanol (DOPAC: 1.8±0.2 pg/µg; HVA: 2.6 pg/µg) had the highest levels in the PFC, and did not differ from each other. Enriched animals not receiving ethanol had the lowest levels (DOPAC: 1.6±0.1 pg/µg; HVA: 2.2±0.1 pg/µg). These data indicate that enrichment decreased these neurochemicals in the PFC, and ethanol self-administration seemed to increase the levels so that they were equal with those of isolated animals. Ethanol did not seem to have an effect in isolated rats. 5-HT (Figure 74) was the greatest in the PFC of isolated animals without ethanol (2.6±0.3 pg/µg). Enriched animals receiving ethanol (1.8±0.1 pg/µg), isolated animals receiving ethanol (1.7±0.2 pg/µg), and enriched animals without ethanol (1.6±0.1 pg/µg) had the lowest amount of serotonin in the PFC, and did not appear to differ from each other. Ethanol decreases serotonin in isolated animals, but appears to have no effect in enriched animals (which already have lower

serotonin levels). The pattern that emerged was similar for DOPAC and HVA, but 5-HT was slightly different, which makes sense because DOPAC and HVA are metabolites of DA (a catecholamine), but 5-HT is an indoleamine. Using the univariate ANOVA, the ethanol x housing interaction was lost for DOPAC, but the interaction remained for HVA and 5-HT. The effect of housing also remained significant for 5-HT.

In summary, only one main effect was found in the prefrontal cortex – enrichment decreased the level of serotonin (I > E). There also was an alcohol x housing interaction for DOPAC and HVA (A E > NA I > A I > NA E), and serotonin (NA I > A E > A I > NA E). This slightly different effect between DOPAC, HVA, and serotonin is likely because DOPAC and HVA are catecholamines and serotonin is an indoleamine. In the PFC, as well as the NAc and VTA, there appear to be differences in findings depending on the method of statistical analysis.

Correlations between Neurochemical and Behavioral Variables

To determine whether any of the behaviors observed in this experiment can be explained by the neurochemical findings, correlational analyses were run. The correlational analyses are presented in a similar order as the results for the behavioral variables discussed above: ethanol consumption in the 2BC paradigm, water consumption, food consumption, open field activity (horizontal and total distance, followed by vertical activity, then center time), and concluding with the forced swim test. Rotarod performance and ethanol consumption in the

operant LSA paradigm did not correlate with any neurochemical data, and are not presented.

Ethanol Consumption in the 2BC Paradigm

Fluid consumption in the 3% Ethanol phase of the 2BC paradigm was negatively correlated with 5-HIAA in the NAc ($r = -0.387$; $p<0.001$) and 5-HT in the PFC ($r = -0.258$; $p<0.05$). During the 6% Ethanol phase, fluid consumption was positively correlated with 5-HT in the NAc ($r = 0.324$; $p<0.01$) and negatively correlated with NE in the PFC ($r = -0.311$; $p<0.05$). Fluid consumption in the 12% Ethanol phase, fluid consumption was positively correlated with NE in NAc ($r = 0.247$; $p<0.05$) and 5-HIAA in the PFC ($r = 0.283$; $p<0.05$). During the last 12% Ethanol phase (right before euthanasia), fluid consumption in the 2BC was positively correlated with NE in the PFC ($r = 0.373$; $p<0.05$).

From these data, it appears that fluid consumption in this paradigm is generally associated with changes in NE, 5-HT, and 5-HIAA in the nucleus accumbens and the prefrontal cortex. While the direction of these correlations were slightly different depending on the phase within the 2BC paradigm, it is clear that changes in these three neurochemicals within these brain areas are somehow associated with ethanol consumption. Neurochemical changes within the VTA do not appear to be associated with ethanol consumption in this paradigm.

Water Consumption

Water consumption was negatively correlated with 5-HT during the 2BC phase ($r = -0.255$; $p<0.05$) and 5-HIAA during the operant LSA phase ($r = -0.273$; $p<0.05$) in the nucleus accumbens. Baseline water consumption was negatively correlated with 5-HT in the ventral tegmental area ($r = -0.300$; $p<0.05$). Further, there were negative correlations between water consumption in the 2BC and DA ($r = -0.258$; $p<0.05$), DOPAC($r = -0.253$; $p<0.05$), HVA ($r = -0.271$; $p<0.05$), and 5-HT ($r = -0.312$; $p<0.05$) in the PFC. It appears that lower levels of indoleamines in the NAc and VTA, and lower levels of catecholamines in the PFC were associated with increased water consumption.

Food Consumption

Food consumption was not associated with neurochemical changes in the nucleus accumbens, but food consumption during the operant LSA phase was positively correlated to NE in the VTA ($r = 0.273$; $p<0.05$). Food consumption did not correlate with neurochemicals in the PFC.

Open Field Activity

Horizontal Activity and Total Distance Traveled. Baseline horizontal activity was negatively correlated with 5-HIAA in the NAc ($r = -0.353$; $p<0.01$), and baseline total distance was negatively correlated with DOPAC in the same brain region ($r = -0.248$; $p<0.05$). Baseline activity was not associated with neurochemical changes in the VTA or PFC. During the 2BC phase, both

horizontal activity ($r = 0.324$, $p<0.05$) and total distance traveled ($r = 0.321$; $p<0.05$) were positively correlated with NE in the VTA. Total distance traveled also was positively correlated with 5-HIAA ($r = 0.282$; $p<0.05$) in this brain region. No correlations were found for activity in the 2BC phase and neurochemicals in the NAc and PFC. Horizontal activity and total distance traveled during the operant LSA phase did not correlate with any neurochemicals from any of the three brain regions examined.

These correlations suggest that general movement and activity have a negative relationship with neurochemicals in the nucleus accumbens, but a positive relationship with neurochemicals in the VTA. No relationship appears to exist between activity and neurochemical levels in the PFC.

Vertical Activity. During the Baseline phase, vertical activity (*i.e.,* exploratory activity) was negatively correlated with DOPAC in the NAc ($r = -0.257$; $p<0.05$). No other relationship was found for baseline vertical activity and other neurochemicals in the NAc, or neurochemicals in the VTA or PFC. Vertical activity in the 2BC was positively correlated with 5-HIAA in the VTA ($r = 0.295$; $p<0.05$). No other relationships with the neurochemical data emerged. During the operant LSA phase, vertical activity was associated with decreased DOPAC in the VTA ($r = -0.253$; $p<0.05$). Again, no other relationships between vertical activity and neurochemical levels appeared. These data suggest that increased vertical or exploratory activity is associated with decreased levels of DOPAC in the reward pathway (*i.e.,* NAc and VTA), and increased levels of 5-HIAA in the VTA.

Center Time. Time spent in the center of the open field apparatus did not correlate to any of the neurochemicals in any of the brain regions during the Baseline phase. Center time during the 2BC phase was negatively correlated with HVA in the PFC ($r = -0.261$; $p<0.05$). During the operant LSA phase, center time was positively correlated with 5-HIAA in the NAc ($r = 0.297$; $p<0.05$). No other correlations emerged during the 2BC or operant LSA phases for open field center time. Taken together, it appears that increased time spent in the center of the open field (*i.e.,* decreased anxiety) is associated with decreased HVA in the PFC and increased 5-HIAA in the NAc.

Summary of Open Field Correlations. Increased overall locomotor movement and activity (*i.e.,* horizontal activity and total distance travel) is associated with increased neurochemicals in the NAc, but decreased levels in the VTA for catecholamines and indoleamines. Conversely, increased exploratory activity (*i.e.,* vertical activity) and decreased anxiety (*i.e.,* increased center time) are associated with decreased catecholamines and increased indoleamines, regardless of brain region.

Forced Swim Test

Immobility in the forced swim test during the 2BC phase was not correlated with neurochemical levels in any of the three brain regions examined. However, number of immobile episodes in the forced swim test during the operant LSA phase was negatively correlated with NE in the VTA ($r = -0.278$; $p<0.05$) and positively correlated with DA in the PFC ($r = 0.294$; $p<0.05$). These

findings suggest that increased depressive-like behavior (*i.e.,* immobility) is associated with increased DA in the PFC and decreased NE in the VTA.

Discussion

The present experiment was designed to examine individual and combined effects of stress and social enrichment on alcohol consumption, and also to examine the effects of alcohol consumption, stress, and social enrichment on brain neurochemistry, anxiety, and depression in rats. It was hypothesized that: **(1) stress will** (a) increase alcohol self-administration (Piazza and LeMoal, 1998; Goeders, 2003; Ahola et al., 2006; Helzer et al., 2006), (b) increase biological and psychological stress responses (Selye, 1936), and (c) increase effects of alcohol on biological and psychological stress responses (Conger, 1956; Sher et al., 2007); **(2) alcohol will** (a) increase biological stress responses (e.g., stress hormone levels) (Kreek and Koob, 1998) and (b) attenuate psychological stress responses (e.g., indices of anxiety) (Conger, 1956; Sher et al., 2007); and **(3) social enrichment will** (a) decrease alcohol self-administration (Deatherage, 1972; Parker and Radow, 1974; Schenk et al., 1990; Wolffgramm, 1990; Deehan et al., 2007), (b) attenuate biological and psychological stress responses (Singer et al., 2005; Yang et al., 2006), and (c) attenuate effects of alcohol on biological and psychological stress responses (Hellemans et al., 2005).

To address these hypotheses, a 2 (alcohol or no alcohol) x 2 (stress or no stress) x 2 (isolated vs. triple-housing) full-factorial design (Table 1) was used with alcohol, stress, and housing condition as independent variables. Dependent variables were biological (levels of dopamine, serotonin, and their metabolites from NAc, VTA, and PFC tissue samples, serum alcohol content, serum corticosterone concentration, and body weight) and behavioral (alcohol consumption in the two-bottle choice paradigm and an operant conditioning paradigm, open field locomotor activity to assess general movement and anxiety-related behavior, rotarod performance to assess balance and coordination, immobility in the forced swim test to assess the depression symptom of learned helplessness, and food and water consumption). This section highlights the major experimental findings, validates the independent variables (to ensure that the manipulations caused the desired effects), discusses the major findings relevant to each independent variable, considers study limitations, and suggests future directions. The conclusion summarizes the major findings and puts them in the context of the current literature.

Overview of Major Experimental Findings

Stress and alcohol are two environmental stimuli that are known to affect behavior in a variety of ways. The present experiment confirmed this fact, and expanded on it. The major findings of this experiment were: (1) stress is disruptive; (2) alcohol is disruptive; (3) social housing has an effect on neurochemistry and behavior which is not necessarily beneficial or detrimental.

These findings were consistent across many of the behavioral variables, including food and water consumption, alcohol self-administration, rotarod performance, open field activity, and the forced swim test. The new findings from this experiment that add to the literature were: (1) stress and alcohol act in a non-additive, non-multiplicative way, that is, they seem to cancel each other out for depressive-like behavior; (2) alcohol consumption may be different depending on the method of self-administration used (*i.e.,* 2BC vs. operant LSA); and (3) environment/housing condition matters. These findings and interpretations are discussed in detail below, but first it is necessary to validate the independent variables.

Validation of Independent Variables

The chronic stress paradigm (predator stress + unpredictable stimuli for 10 mins per day, 3x per week) was validated with the dependent variables of serum corticosterone concentration (a biomarker of the HPA axis) and body weight (*e.g.,* Shafer, 2005; Berger, 2009; Perry, 2009; Hamilton, 2010; Long, 2010). As expected, rats in the stressed condition weighed less and had higher levels of serum corticosterone at the conclusion of the experiment, which was 3-4 days after the last stress exposure. Based on these findings, which are comparable to previous findings from our laboratory (Faraday, 2002; Faraday et al., 2005; Shafer, 2005; Berger, 2009; Perry, 2009; Hamilton, 2010; Long, 2010), the stress paradigm used in this experiment did indeed induce stress. Interestingly, social enrichment also increased corticosterone. It appears that social housing in itself

is arousing, although not necessarily stressful *per se*. The mostly likely cause for this finding is that when the rats are socially housed, they are more active in their home cages than single-housed rats, which increases circulating corticosterone. Because of this finding, it is important to keep in mind that housing conditions need to be considered when examining effects of stress on physiological stress responses.

Along with the stress paradigm, the alcohol self-administration paradigm needed to be validated by a dependent variable. Both biological and behavioral measures were used. Serum ethanol concentration was assayed at the conclusion of the experiment, and rotarod performance was measured during each of the alcohol self-administration phases (*i.e.,* 2BC and operant LSA phases). Rotarod is a simple measure of balance and coordination, behavior that can be detrimentally affected by alcohol consumption. Rats that were assigned to the alcohol group performed more poorly on this task than did rats assigned to the no alcohol group. Based on this finding, it is reasonable to interpret that the animals assigned to the alcohol group were affected by the alcohol they consumed. Importantly, animals in the alcohol group had higher levels of serum ethanol than did animals in the no alcohol group. Taken together, it is certain that the rats in the stress condition were stressed, and rats in the no stress condition were less stressed (especially in the isolated condition); rats in the alcohol condition were consuming alcohol, and animals in the no alcohol condition did not have any access to alcohol. Therefore, the independent variables were manipulated as predicted and any effects or interactions on the

dependent variables, therefore, may be considered meaningful. The interpretations of findings are presented below, first discussing effects of stress, then effects of alcohol, stress x alcohol interactions, and next considering housing effects. In each section, the dependent variables are presented in the same order as in the Results section.

Stress Effects

For most of the dependent variables examined in this experiment (including water consumption, food consumption, activity, depressive index, and some neurochemistry), stress had a detrimental or disruptive effect. However, two variables (*i.e.,* rotarod performance and alcohol consumption) were differentially affected by stress. Rotarod performance was enhanced in rats that were stressed, and there was no effect of stress to alter alcohol consumption. It seems that for rotarod, the moderate stressor in this experiment pushed rats to the peak of their performance capabilities, but was not severe enough to cause a detriment. This idea is visualized with the Yerkes-Dodson curve (see figure, pg. 49; Yerkes and Dodson, 1908) Perhaps a more severe stressor would have pushed the rats further along the inverted-U shaped performance curve, and impaired the animals' ability to perform.

Aside from this one variable, the disruptive effects of stress are quite clear for the other dependent variables. Stress decreased fluid consumption, both in the 2BC task and in normal daily water intake over the course of the experiment. Decreased fluid intake could lead to dehydration and other negative

consequences, including impaired cognitive performance (Ritz and Berrut, 2005; Kempton et al., 2010). While water intake decreased, food consumption increased in stressed rats as compared to non-stressed rats, even though stressed rats weighed less (cf. Validation of Independent Variables, pg, 81, for brief discussion of stress and body weight). There are several possible explanations for this phenomenon. Perhaps stress increased food metabolism, leading the rats to consume more food. Another possibility is that stressed animals were more active than non-stressed animals, leading them to eat more. A third possibility is that the animals were coping with stress by eating more and that faster metabolism or increased activity is unrelated to the feeding behavior. Based on findings from open field activity, stress only increased activity in isolated animals, but actually decreased activity in socially enriched animals. Therefore, activity cannot be the sole explanation for increased food consumption and lower body weight in the stressed animals.

While stress increased overall activity in the open field locomotor measure, stress did not decrease open field center time (an index of anxiety). In fact, stress did not affect anxiety at all according to this measure. According to the human literature (*e.g.,* Baum et al., 1993), stress increases anxiety, so it was expected that the same result would emerge in the current experiment. There are several possible explanations for this discord. First, it is possible that although this stress paradigm causes an increase in circulating corticosterone and some behavioral effects, it does not increase anxiety. A second possibility is that this index of rodent anxiety is not sensitive enough to observe effects of the

stress paradigm used in this experiment. The only way to determine whether either explanation is correct would be to run another experiment using the same stress paradigm but testing anxiety with an alternate measure, such as the elevated plus maze (Pellow et al., 1985; Hogg, 1996; Elliott et al., 2004; Berger, 2009). Although stress did not seem to increase anxiety, it did increase depressive-like behaviors in the forced swim test, especially for animals that were in the no alcohol condition.

Many reports in the literature have addressed the question of what effects stress has on the brain (Richardson et al., 1974; Moyer et al., 1977; Deutch and Roth, 1990; McEwen, 2000; Meloni et al., 2008; Mo et al., 2008). In this experiment, stress only seemed to affect one of the three brain regions examined, the nucleus accumbens. In fact, stress decreased norepinephrine (NE) and the serotonin metabolite 5-hydroxyindoleacetic acid (5-HIAA). The decrease in accumbal NE is interesting because it is well known that stress increases circulating NE and epinephrine through sympathetic nervous system activation (Guyton and Hall, 2000a). However, several reports have shown that stress decreased NE in the NAc (*e.g.,* De La Garza and Mahoney, 2004), but it's possible that this finding may be the result of the rat strain (Wistar). A different study using Sprague-Dawey rats found increased NE in the NAc after stress (Cenci et al., 1992).

Similar to NE, 5-HIAA was decreased in the NAc. This finding may mean that serotonin was reduced (though not statistically significant), leading to a reduction in its metabolite, 5-HIAA. If this is true, then it makes sense that stress

reduces the “feel-good,” calming neurochemical, serotonin, and why there was an increase in depressive-like behaviors in stressed animals. Conversely, it could be that although serotonin levels remained the same, its metabolism to 5-HIAA was reduced. If the second explanation were true, then serotonin would build up (which was not seen). This process does not make sense in the context of the current results because decreased anxiety and depression would have been expected with increased serotonin, so it leads one to believe that the former idea is more likely.

With regard to the experimental hypotheses specifically about stress, results are mixed. **Hypothesis 1a** (stress will increase alcohol self-administration) **was not confirmed** and **Hypothesis 1b** (stress will increase biological and psychological stress responses) **was partially confirmed**. Stress appeared to have no effect on alcohol consumption, increased the biological stress response (corticosterone levels), and increased one of the measured psychological stress responses (depression) but had no effect on anxiety. Overall, it appears that stress has a disruptive effect on neurochemistry and behavior, but it did not cause increased alcohol consumption in this experiment.

Alcohol Effects

Similar to the effects of stress, alcohol consumption was somewhat disruptive to some of the physiological and behavioral measures examined, but had no effect on other variables. Alcohol consumption did not appear to be beneficial on its own. Alcohol did not alter the physiological stress response

variables of serum corticosterone or body weight. This finding suggests that the relationship between stress and increased alcohol consumption is not the result of a reduction or alleviation of physiological stress (as measured by increased corticosterone). Further, there were no differences in food or water consumption between animals that drank alcohol and those that did not, showing that animals did not drink alcohol as a replacement for calories that would have normally been obtained by eating food, or because they were thirsty. This finding is consistent with the interpretation that the rats in the alcohol group drank alcohol because they were motivated to do so. Alcohol did not affect open field locomotion in any way, meaning that reduced performance of alcohol rats on the rotarod task was the result of disrupted balance and coordination, and not changes in gross movement *per se*. Alcohol consumption alone did not alter depressive-like behavior in the forced swim test, but there were interesting stress x alcohol interactions (described in the following section). Alcohol did not affect neurochemical levels in the nucleus accumbens or prefrontal cortex, but alcohol did increase DA, HVA, and NE in the VTA. The increase in DA and its metabolite in the VTA was expected because this brain region is part of the mesolimbic dopamine system and implicated in reward and reinforcement, including alcohol and drug reward (Gonzales et al., 2004; Koob and Le Moal, 2006a; Koob and Le Moal, 2006b; Feltenstein and See, 2008; Anselme, 2009). This finding suggests that alcohol was reinforcing to the animals that had access to it.

Stress-Alcohol Interactions

It is well known that stress and alcohol use tend to occur together (Le et al., 1998; Le et al., 2000; Hansson et al., 2006; Helzer et al., 2006; Fullgrabe et al., 2007; Sher et al., 2007; Sinha and Li, 2007; Sterud et al., 2007; Zimmerman et al., 2007; Sommer et al., 2008; Grunberg et al., 2010), and there are several explanations for this relationship. First, it is possible that alcohol actually reduces the physiological stress response (Dai et al., 2007). In the current experiment, this reduction was not the case. Alcohol appears to have had no effect on the physiological indices of stress examined (*i.e.,* serum corticosterone, body weight).

A second possibility is that alcohol reduces psychological effects of stress, such as anxiety and depression (Conger, 1956; Khantzian, 1985; Sher et al., 2007), meaning that animals (including people) may use alcohol to manage or alleviate these psychological effects. Based on the rodent behavior index of anxiety used in this experiment (*i.e.,* time spent in the center of an open field), this result did not occur. Stress did not increase anxiety, alcohol did not decrease anxiety, and there was no stress x alcohol interaction, suggesting that this second possibility might not be true. However, as described above (under "Stress Effects"), open field center time is not the only rodent index of anxiety, and it is possible that this measure was not sensitive enough to observe stress or alcohol effects. The only way to confirm this finding would be to use an alternate anxiety index such as the elevated plus maze (Pellow et al., 1985; Hogg, 1996; Elliott et al., 2004; Berger, 2009) While stress and alcohol did not appear to

affect anxiety, there was a clear stress x alcohol interaction for depression. Stress alone and alcohol alone increased immobility in the forced swim test (*i.e.,* increased depression); however, stress and alcohol together reduced depression. In fact, the stressed alcohol group was quite similar to the non-stressed no alcohol group on this depression index. If this finding holds true for humans, then it makes sense why people drink when they are under stress – it reduces depression under stress. Perhaps, low-moderate alcohol use is beneficial when one is under stress, but the same alcohol consumption while unstressed could have negative consequences.

An alternative explanation for increased alcohol use under stress is that alcohol alters stress-induced neurochemical changes. After examining dopamine, norepinephrine, serotonin, and metabolites in several brain regions, it appears that there are several effects of stress and alcohol (as described in the preceding sections), but it is not yet clear how these neurochemical effects may help to explain the stress-alcohol use relationship. It is possible that stress and alcohol may have a combined effect on other brain regions (*e.g.,* amygdala, hippocampus, insula) or on other neurochemicals (*e.g.,* GABA, glutamate, endogenous opioid peptides) related to stress and drug use (Del Arco and Mora, 2001; Braga et al., 2004; Kreek, 2007; Jiang et al., 2008). However, based on the results described here, this explanation cannot be accepted with certainty.

A final possibility to explain the stress-alcohol consumption relationship is that alcohol alters attention by diverting it away from the stressor(s). While it is possible to measure attention in rodents using tasks such as inhibition of the

startle response by a prepulse or the 5-choice serial reaction time task (Sher et al., 2007), it is difficult to determine whether animals are engaged in “thinking” about a stressor. The best way to address this explanation is by using human subjects.

From these findings, it is clear that low to moderate amounts of alcohol reduces stress-induced depression, but does not affect stress-induced physiological responses, anxiety, or neurochemical changes in the nucleus accumbens, ventral tegmental area, or prefrontal cortex. These data support the explanation that people under stress may drink alcohol to cope with stress-induced depression, which supports the self-medication hypothesis (Khantzian, 1985; Hall and Queener, 2007). It is important to point out that low to moderate alcohol use may be beneficial to alleviate stress-induced depression, but heavy alcohol use has many detrimental effects (such as sedation, memory impairment, slowed reaction time, ataxia and imbalance, dizziness, blurry vision, slurred speech, dizziness and nausea, potential coma and death). Therefore, physicians should be aware of whether their patients are using alcohol for this purpose, and monitor how much alcohol their patients are drinking. If patients are staying within the acceptable (and potentially beneficial) low-moderate dose range, physicians may even want to recommend continued alcohol consumption. However, if this behavior leads to heavy or binge drinking, then the physician needs to help the patient understand why he or she is using alcohol in this way (*i.e.,* self-medication to reduce stress-induced depression), reduce alcohol intake, and find other ways to cope with stress.

To revisit the stress-alcohol hypotheses, 2a (alcohol will increase the biological stress response) and 2b (alcohol will decrease the psychological stress response), it appears that **2a was not confirmed, but 2b was partially confirmed**. Alcohol did not reduce the increase in serum corticosterone that occurred in stressed rats. Alcohol did not reduce anxiety-like behavior as measured by open field center time, but it did decrease stress-induced depression as measured by the forced swim test.

Housing Effects

Environmental conditions such as stress and access to alcohol can have profound effects on physiology, neurochemistry, and behavior. But social environment has just as notable effects, which have been found in the human literature, animal literature, and this experiment. Rats housed in groups of three had higher levels of circulating corticosterone than did rats housed singly, confirming that group housing is arousing for male rats (Brown and Grunberg, 1995). Social enrichment also increased overall food and water intake, as well as alcohol or water intake in the 2BC paradigm. This finding is interesting because it supports the idea that many organisms eat more in the presence of others, including humans (de Castro et al., 1990; de Castro, 1991; Clendenen et al., 1994; de Castro, 1994, 1995; Feunekes et al., 1995; Pliner et al., 2006; Drewett, 2007), dogs (Ross and Ross, 1949a; Ross and Ross, 1949b; James, 1953), and even chicks (Tolman, 1965).

While enriched rats in the alcohol group drank more alcohol than those in the isolated group, this effect was reversed in the operant LSA paradigm. In the operant chambers, rats in the isolated housing condition drank more than did socially enriched rats. This finding was particularly true for stressed isolated rats. It is possible that when enriched animals were placed into the operant chambers individually, the social facilitation of alcohol consumption in the 2BC paradigm was lost. Another possibility for this change is that the enriched animals habituated more quickly (see below) to the operant chambers and, therefore, were less active. However, there was no effect of enrichment on lever pressing for water, so the most reasonable explanation for the reversal of housing effects from the 2BC paradigm to the operant LSA paradigm is because the social facilitation of alcohol consumption in the home cage was lost when socially enriched animals were individually placed into the operant chambers.

Enrichment has a huge effect on all activity in the open field environment. Isolated animals had greater horizontal activity, vertical activity, spent more time in the center of the apparatus, and traveled a further distance. Conversely, socially enriched animals habituated faster to the environment than did isolated animals. These results replicated previous findings from our laboratory (*e.g.,* Elliott and Grunberg, 2005; Long, 2010). This effect of enrichment on activity suggests that socially enriched animals have higher home cage activity from cage mate interactions, but being placed alone in another chamber is uninteresting and, possibly, boring . However, for rats housed alone, being placed in the new environment for an hour is interesting and affords an

opportunity to explore, which explains why they have greater activity in the open field than socially enriched animals. In the forced swim test, enriched animals had greater immobility than isolated animals, indicating greater depressive-like behavior. It is possible that this was a more stressful situation for enriched animals than for isolated animals because enriched animals were removed from their cage mates and placed alone in the aversive environment.

The major effect of housing on brain neurochemistry was that enrichment increased dopamine and HVA in the nucleus accumbens. This finding is interesting because it suggests that presence of others is rewarding. While there have been many reports suggesting that social and physical environment can alter the rewarding effects of drugs (Bowling et al., 1993; Bowling and Bardo, 1994; Bardo et al., 1995; Bardo et al., 2001; Rahman and Bardo, 2008), this may be the first experiment to examine the individual effects of housing condition on dopamine in the mesolimbic reward pathway.

With regard to the hypotheses about enrichment effects, **Hypothesis 3a** (social enrichment will decrease alcohol self-administration) **was partially confirmed, Hypothesis 3b** (social enrichment will attenuate biological and psychological stress responses) **was not confirmed**, and **Hypothesis 3c** (social enrichment will attenuate alcohol's effects on biological and psychological stress responses) **was not confirmed**. Social enrichment increased alcohol consumption (as well as food and water consumption) in the 2BC paradigm, but decreased alcohol consumption in the operant LSA paradigm. This finding may be the result of social facilitation within the group housing of the 2BC paradigm,

that disappeared when animals were individually placed into the operant chambers. Social enrichment did not attenuate stress responses. In fact, enrichment (as well as stress) increased serum corticosterone levels, and enriched animals were more likely to exhibit stress-induced depressive behavior. Social enrichment could not attenuate effects of alcohol on several of the stress responses because alcohol did not affect serum corticosterone or anxiety-like behavior in this experiment. However, it appeared that alcohol attenuated the stress-induced depression in enriched animals, as opposed to enrichment attenuating alcohol's effect on stress-induced depression.

Relationships between Neurochemistry and Behavior

There were some weak correlations ($|r| < 0.400$) between certain neurochemicals and certain behaviors, but clear patterns were not apparent. There appeared to be effects of stress, alcohol, and housing on many of the behaviors, and many of the neurochemicals, but it is not certain whether any one neurochemical or group of neurochemicals underlies any particular behavior or group of behaviors. There are two interpretations for this finding. First, it is possible that there really is no pattern. A second explanation for these vague findings may be that the neurochemical procedures used in this experiment was not robust enough to detect neurochemical differences that were meaningful with regard to the behavior tasks. Perhaps another method (*e.g.,* microdialysis) would have yielded better results. A third explanation is that subtle differences in neurochemical levels across these brain regions are responsible for the

behaviors examined in this experiment, but the statistical analyses used were not sophisticated enough to elucidate the pattern. Using a more advanced statistical approach (*e.g.,* Canonical discriminitive analysis, in which multiple independent variables are considered with subtle changes across multiple dependent variables) could be worthwhile.

Study Limitations

While there were clear effects of stress, alcohol, and housing on most of the dependent variables, there remain some limitations (including theoretical, methodological, and generalizability limitations) to this experiment that need to be addressed. The most clinically-relevant way to study the effects of stress and social environment on alcohol consumption and alcohol's effects in the human brain would be to use human subjects. However: (1) true experiments cannot be conducted in humans that manipulate stress and alcohol intake; (2) history of alcohol or other drug use is difficult to control; (3) social environment is difficult to control and manipulate in humans; (4) stressors outside the experimental conditions may confound results; and (5) neurochemical levels cannot be assessed in specific brain loci (unless tissue is available in a postmortem tissue bank). Animal research is valuable to determine causation, unrelated variables can be controlled, and biochemical changes in the brain can be measured in response to different manipulations.

One limitation to the present experiment is that ethanol was individually self-administered only by isolated animals during the 2-bottle choice phase, and by all

animals in the operant phase. These methods may limit the generalizability of the results because humans often consume alcohol in social settings. In addition, the order of these two paradigms remained the same for all subjects, that is, all subjects were exposed to the 2BC paradigm, followed by the operant LSA paradigm. While this was a deliberate methodological decision based on the literature (Le et al., 1998; Le et al., 2000), it could have created an order effect that confounded the data. Further, only a single method was used to induce stress in this experiment (*i.e.,* predator/unpredictable stress). To expand the generalizability of the findings, this experiment used several mild to moderate stressors (*i.e.,* predator stress and unpredictable stressors), but these stressors did not affect alcohol consumption as expected from the previous literature. The generalizability of the findings also was limited by using only males as subjects. Males were chosen for logistical purposes because they are more likely to binge and heavy drink, and they are more likely to suffer adverse consequences from drinking alcohol.

Two methodological limitations became apparent after data analysis, and may have affected the ability to interpret the data. First, there seemed to be a problem with the serum ethanol assay. Although the assay demonstrated that rats with access to alcohol had higher levels of ethanol in the blood than did rats without access, levels of ethanol were “apparent” in rats without access to alcohol based on this assay. This result seems impossible – rats that had no access to alcohol should not have alcohol in their blood. However, this was a spectrophotometric assay, and perhaps serum samples that were redder or more

turbid had confounded results. This assay was chosen based on reports in the literature (*e.g.,* Poklis and Mackell, 1982; Webb et al., 2002; Liao et al., 2007), but these reports did not include "No alcohol" groups, only groups that received different doses of ethanol. Therefore, this assay may be good for determining serum ethanol concentration for animals that were administered alcohol, but it may not be good for comparing alcohol groups to no alcohol groups. The second major methodological limitation was that the neurochemicals were assayed in *post-mortem* brain tissue as opposed to the active release of these neurochemicals during behavioral responding. Using methods such as microdialysis or voltammetry would examine moment-to-moment differences in neurochemical levels during alcohol consumption or stress induction, but the setups are expensive, they have low throughput, and the surgery and techniques are somewhat difficult to learn. For the purposes of this doctoral dissertation research, *post mortem* analyses were the best option, and these analyses often to yield results similar to microdialysis results.

Future Directions

The first future direction for the present research would be to repeat the experiment in female animals. Much research on stress, social enrichment, drug self administration, drug action, and brain neurochemistry has demonstrated sex differences (Holck et al., 1937; Griffin et al., 1989; Lex, 1991; Brown and Grunberg, 1995, 1996; Klein et al., 1998; Faraday, 2002; Webb et al., 2002; Elliott et al., 2003; Elliott et al., 2004; Faraday et al., 2005; Wagner et al., 2005;

Mitsushima et al., 2006; Pena et al., 2006; Dalla et al., 2008; Berger, 2009; Perry, 2009; Long, 2010), so it is important for females to be considered as well as males. To avoid any possible order effects or age effects from always having the 2BC paradigm precede the operant LSA paradigm (and the animals always being younger in the 2BC and older in the operant LSA), the experiment could be repeated with the order of alcohol self-administration paradigms presented in the reverse order. It would also be valuable to compare effects of stress, alcohol, and environmental enrichment on rats of different ages (*e.g.,* adolescents, young adults, older adults) to determine how age may play a role in any of the effects seen from this experiment. The next step would be to examine different levels of stress on alcohol consumption and effects on the brain and behavior. Stress is associated with increased alcohol consumption in humans (Conger, 1956; Piazza and LeMoal, 1998; Ahola et al., 2006; Helzer et al., 2006; Schroder and Perrine, 2007; Sher et al., 2007) and rodents (Breese et al., 2005; Hansson et al., 2006; Fullgrabe et al., 2007), but in this experiment, stress did not alter alcohol intake. Even though this stress paradigm was stressful (increased corticosterone and depressive-like behavior), it is possible that the moderate stressor was not severe enough to induce the animals to drink more alcohol.

In this experiment, behavior and neurochemical measures were taken sequentially, and only correlational analyses could be done after all data were gathered. For example, there is no way to know whether changes in behavior (*i.e.,* increased alcohol consumption) actually increased dopamine in the nucleus accumbens, or if increased basal levels of dopamine made certain animals more

susceptible to increased alcohol consumption. Therefore, using another method of measuring brain neurochemicals (*e.g.,* microdialysis) would allow one to examine basal neurochemical levels, and neurochemical changes in response to stress induction or alcohol consumption. Combining the variables of stress, social enrichment, and access to alcohol in a microdialysis paradigm would allow for causal conclusions to be made. In addition, more sophisticated statistical analyses could be useful in examining the relationships between neurochemicals and behavior.

Aside from neurochemistry and behavior, other neuroscience techniques would be valuable for further exploring effects of stress, alcohol, and social enrichment on the brain reward system. Imaging studies, similar to those done by Volkow and colleagues (2004) could provide interesting data on activation of specific brain loci before and after stress induction or alcohol self-administration. In addition, examining effects of these variables on dopamine, serotonin, or glucocorticoid receptor density and sensitivity in these brain regions would help to clarify what functional changes in the brain occur in response to these variables.

Finally, it is important to try to understand how these variables might interact and play a role in human alcohol consumption. Many human studies try to focus efforts on one particular variable (such as effects of stress on alcohol consumption), but the results of the combined variables in the present experiment are interesting and important. Here it was determined that stress was disruptive, alcohol was disruptive, and alcohol seemed to buffer the effects

of stress on depressive-like behavior. It would be wise to design human experiments to test the findings from the present animal research, to find out how well the findings hold up. If so, perhaps physicians should be telling patients that small to moderate amounts of alcohol should be used in order to reduce stress and stress-induced depression.

Summary and Conclusion

In summary, stress and alcohol consumption each can be detrimental or disruptive to biology and behavior, but together, alcohol consumption buffers or reduces stress-induced depression. Further, social housing conditions alter brain neurochemistry, circulating corticosterone, and many behaviors. Overall, the results suggest that stress is disruptive and alcohol can alleviate the negative effect of stress on mood. However, if one is not stressed, alcohol alone can be disruptive. So alcohol should be avoided when not under stress. These results are particularly interesting and important if they extrapolate to humans. The effects of housing are particularly valuable for rodent research in neuroscience. Often, investigators fail to report animal housing conditions when writing up results for publication. However, this experiment clearly demonstrated that housing can affect behavior and neurochemical levels in the brain. Therefore, it is important to consider housing conditions when publishing findings, replicating findings, and designing future experiments. Further, it is apparent that combining neurochemical and behavioral methods are valuable in neuroscience research, but the statistical tools used to evaluate both types of methods in the same

experiment are still in infancy. In the future, it would be wise to consider more sophisticated statistical methods for analyzing the two types of data together in an effort to clearly determine relationships between the brain and behavior.

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Appendix A:

Table Appendix

Table 14 – ANOVA Table for Serum Corticosterone

Source	Sum of Squares	df	Mean Square	F	Sig.
Ethanol	46675	1	46675	1.518	0.223
Stress	172447	1	172447	5.609	0.021
Housing	527345	1	527345	17.154	0.000
Ethanol * Stress	8847	1	8847	0.288	0.594
Ethanol * Housing	20391	1	20391	0.663	0.419
Stress * Housing	259329	1	259329	8.436	0.005
Ethanol * Stress * Housing	311	1	311	0.010	0.920
Error	1906012	62	30742		

Table 15 – Repeated-Measures ANOVA Table for Body Weight, Within-Subject Effects

Source	Sum of Squares		df	Mean Square	F	Sig.
phase	Sphericity Assumed	2311569	2	1155785	5875.552	0.000
	Greenhouse-Geisser	2311569	1.205	1918710	5875.552	0.000
phase * Housing	Sphericity Assumed	479	2	240	1.218	0.300
	Greenhouse-Geisser	479	1.205	398	1.218	0.283
phase * Stress	Sphericity Assumed	1316	2	658	3.345	0.039
	Greenhouse-Geisser	1316	1.205	1092	3.345	0.064
phase * Ethanol	Sphericity Assumed	19	2	10	0.049	0.952
	Greenhouse-Geisser	19	1.205	16	0.049	0.868
phase * Housing * Stress	Sphericity Assumed	325	2	162	0.826	0.440
	Greenhouse-Geisser	325	1.205	270	0.826	0.387
phase * Housing * Ethanol	Sphericity Assumed	358	2	179	0.911	0.405
	Greenhouse-Geisser	358	1.205	297	0.911	0.361
phase * Stress * Ethanol	Sphericity Assumed	744	2	372	1.890	0.155
	Greenhouse-Geisser	744	1.205	617	1.890	0.172
phase * Housing * Stress * Ethanol	Sphericity Assumed	17	2	9	0.044	0.957
	Greenhouse-Geisser	17	1.205	14	0.044	0.876
Error	Sphericity Assumed	23999	122	197		
	Greenhouse-Geisser	23999	73.490	327		

Table 16 – Repeated Measures ANOVA Table for Body Weight, Between Subjects Effects

Source	Sum of Squares	df	Mean Square	F	Sig.
Housing	970	1	970	0.860	0.357
Stress	1157	1	1157	1.026	0.315
Ethanol	232	1	232	0.206	0.651
Housing * Stress	52	1	52	0.046	0.831
Housing * Ethanol	1472	1	1472	1.305	0.258
Stress * Ethanol	3423	1	3423	3.035	0.087
Housing * Stress * Ethanol	809	1	809	0.717	0.400
Error	68806	61	1128		

Table 17 – ANOVA Table for Body Weight during Baseline Phase

Source	Sum of Squares	df	Mean Square	F	Sig.
Housing	5	1	5	0.039	0.844
Stress	90	1	90	0.768	0.384
Ethanol	101	1	101	0.870	0.354
Housing * Stress	31	1	31	0.268	0.606
Housing * Ethanol	55	1	55	0.472	0.495
Stress * Ethanol	126	1	126	1.084	0.302
Housing * Stress * Ethanol	223	1	223	1.913	0.171
Error	7348	63	117		

Table 18 – ANOVA Table for Body Weight during the 2BC Phase

Source	Sum of Squares	df	Mean Square	F	Sig.
Housing	296	1	296	0.731	0.396
Stress	873	1	873	2.154	0.147
Ethanol	142	1	142	0.350	0.556
Housing * Stress	89	1	89	0.219	0.641
Housing * Ethanol	1133	1	1133	2.796	0.099
Stress * Ethanol	1109	1	1109	2.738	0.103
Housing * Stress * Ethanol	218	1	218	0.539	0.465
Error	25521	63	405		

Table 19 – ANOVA Table for Body Weight during the Operant LSA Phase

Source	Sum of Squares	df	Mean Square	F	Sig.
Housing	697	1	697	0.655	0.421
Stress	2256	1	2256	2.122	0.150
Ethanol	184	1	184	0.173	0.679
Housing * Stress	122	1	122	0.115	0.735
Housing * Ethanol	665	1	665	0.626	0.432
Stress * Ethanol	1722	1	1722	1.619	0.208
Housing * Stress * Ethanol	146	1	146	0.137	0.712
Error	65917	62	1063		

Table 20 – ANOVA Table for Serum Ethanol Concentration

Source	Sum of Squares	df	Mean Square	F	Sig.
Ethanol	28532	1	28532	9.588	0.003
Stress	3619	1	3619	1.216	0.274
Housing	10487	1	10487	3.524	0.065
Ethanol * Stress	3691	1	3691	1.240	0.270
Ethanol * Housing	25590	1	25590	8.599	0.005
Stress * Housing	10467	1	10467	3.517	0.066
Ethanol * Stress * Housing	208	1	208	0.070	0.792
Error	181528	61	2976		

21 – Repeated-Measures ANOVA Table for Rotarod Performance, Within-Subject Effects

Source	Sum of Squares		df	Mean Square	F	Sig.
phase	Sphericity Assumed	58408	2	29204	8.816	0.000
	Greenhouse-Geisser	58408	1.678	34816	8.816	0.001
phase * Housing	Sphericity Assumed	5135	2	2567	0.775	0.463
	Greenhouse-Geisser	5135	1.678	3061	0.775	0.443
phase * Stress	Sphericity Assumed	6627	2	3313	1.000	0.371
	Greenhouse-Geisser	6627	1.678	3950	1.000	0.359
phase * Ethanol	Sphericity Assumed	4706	2	2353	0.710	0.493
	Greenhouse-Geisser	4706	1.678	2805	0.710	0.470
phase * Housing * Stress	Sphericity Assumed	405	2	202	0.061	0.941
	Greenhouse-Geisser	405	1.678	241	0.061	0.914
phase * Housing * Ethanol	Sphericity Assumed	6000	2	3000	0.906	0.407
	Greenhouse-Geisser	6000	1.678	3577	0.906	0.392
phase * Stress * Ethanol	Sphericity Assumed	0	2	0	0.000	1.000
	Greenhouse-Geisser	0	1.678	0	0.000	1.000
phase * Housing * Stress * Ethanol	Sphericity Assumed	663	2	331	0.100	0.905
	Greenhouse-Geisser	663	1.678	395	0.100	0.872
Error	Sphericity Assumed	424041	128	3313		
	Greenhouse-Geisser	424041	107.370	3949		

Table 22 – Repeated-Measures ANOVA Table for Rotarod Performance, Between-Subjects Effects

Source	Sum of Squares	df	Mean Square	F	Sig.
Housing	103	1	103	0.015	0.901
Stress	28681	1	28681	4.313	0.042
Ethanol	25632	1	25632	3.855	0.054
Housing * Stress	665	1	665	0.100	0.753
Housing * Ethanol	1171	1	1171	0.176	0.676
Stress * Ethanol	165	1	165	0.025	0.875
Housing * Stress * Ethanol	6048	1	6048	0.910	0.344
Error	425555	64	6649		

Table 23 – ANOVA Table for Rotarod Performance during Baseline Phase

Source	Sum of Squares	df	Mean Square	F	Sig.
Housing	4005	1	4005	0.719	0.400
Stress	13312	1	13312	2.391	0.127
Ethanol	1378	1	1378	0.247	0.621
Housing * Stress	2	1	2	0.000	0.984
Housing * Ethanol	115	1	115	0.021	0.886
Stress * Ethanol	55	1	55	0.010	0.921
Housing * Stress * Ethanol	4340	1	4340	0.779	0.381
Error	356379	64	5568		

Table 24 – ANOVA Table for Rotarod Performance during the 2BC Phase

Source	Sum of Squares	df	Mean Square	F	Sig.
Housing	1058	1	1058	0.273	0.603
Stress	20876	1	20876	5.379	0.024
Ethanol	16320	1	16320	4.205	0.044
Housing * Stress	556	1	556	0.143	0.706
Housing * Ethanol	6161	1	6161	1.587	0.212
Stress * Ethanol	57	1	57	0.015	0.904
Housing * Stress * Ethanol	1267	1	1267	0.326	0.570
Error	248378	64	3881		

Table 25 – ANOVA Table for Rotarod Performance during the Operant LSA Phase

Source	Sum of Squares	df	Mean Square	F	Sig.
Housing	174	1	174	0.046	0.832
Stress	1120	1	1120	0.293	0.590
Ethanol	12641	1	12641	3.304	0.074
Housing * Stress	512	1	512	0.134	0.716
Housing * Ethanol	896	1	896	0.234	0.630
Stress * Ethanol	53	1	53	0.014	0.906
Housing * Stress * Ethanol	1105	1	1105	0.289	0.593
Error	244840	64	3826		

Table 26 – Repeated-Measures ANOVA Table for Ethanol or Water Self-Administration in the 2BC Task, Within-Subject Effects

Source	Sum of Squares		df	Mean Square	F	Sig.
phase	Sphericity Assumed	387.013	3	129.004	4.788	0.003
	Greenhouse-Geisser	387.013	1.822	212.444	4.788	0.012
phase * Housing	Sphericity Assumed	570.201	3	190.067	7.054	0.000
	Greenhouse-Geisser	570.201	1.822	313.002	7.054	0.002
phase * Stress	Sphericity Assumed	129.376	3	43.125	1.601	0.191
	Greenhouse-Geisser	129.376	1.822	71.019	1.601	0.208
phase * Ethanol	Sphericity Assumed	19.628	3	6.543	0.243	0.866
	Greenhouse-Geisser	19.628	1.822	10.774	0.243	0.764
phase * Housing * Stress	Sphericity Assumed	184.177	3	61.392	2.279	0.081
	Greenhouse-Geisser	184.177	1.822	101.101	2.279	0.112
phase * Housing * Ethanol	Sphericity Assumed	93.536	3	31.179	1.157	0.328
	Greenhouse-Geisser	93.536	1.822	51.345	1.157	0.315
phase * Stress * Ethanol	Sphericity Assumed	212.309	3	70.770	2.627	0.052
	Greenhouse-Geisser	212.309	1.822	116.544	2.627	0.082
phase * Housing * Stress * Ethanol	Sphericity Assumed	99.553	3	33.184	1.232	0.300
	Greenhouse-Geisser	99.553	1.822	54.648	1.232	0.294
Error	Sphericity Assumed	4768.932	177	26.943		
	Greenhouse-Geisser	4768.932	107.481	44.370		

Table 27 – Repeated-Measures ANOVA Table for Ethanol or Water Self-Administration in the 2BC Task, Between-Subjects Effects

Source	Sum of Squares	df	Mean Square	F	Sig.
Housing	2323.290	1	2323.290	31.684	0.000
Stress	12.866	1	12.866	0.175	0.677
Ethanol	12.000	1	12.000	0.164	0.687
Housing * Stress	210.272	1	210.272	2.868	0.096
Housing * Ethanol	30.269	1	30.269	0.413	0.523
Stress * Ethanol	10.661	1	10.661	0.145	0.704
Housing * Stress * Ethanol	43.405	1	43.405	0.592	0.445
Error	4326.327	59	73.328		

Table 28 – ANOVA Table for "0%" Ethanol or Water Self-Administration in the 2BC Task

Source	Sum of Squares	df	Mean Square	F	Sig.
Housing	260.558	1	260.558	14.424	0.000
Stress	11.682	1	11.682	0.647	0.424
Ethanol	0.240	1	0.240	0.013	0.909
Housing * Stress	0.099	1	0.099	0.005	0.941
Housing * Ethanol	2.006	1	2.006	0.111	0.740
Stress * Ethanol	11.151	1	11.151	0.617	0.435
Housing * Stress * Ethanol	64.621	1	64.621	3.577	0.063
Error	1101.935	61	18.065		

Table 29 – ANOVA Table for 3% Ethanol or Water Self-Administration in the 2BC Task

Source	Sum of Squares	df	Mean Square	F	Sig.
Housing	463.523	1	463.523	18.483	0.000
Stress	0.001	1	0.001	0.000	0.996
Ethanol	1.399	1	1.399	0.056	0.814
Housing * Stress	23.568	1	23.568	0.940	0.336
Housing * Ethanol	0.015	1	0.015	0.001	0.981
Stress * Ethanol	0.471	1	0.471	0.019	0.891
Housing * Stress * Ethanol	2.701	1	2.701	0.108	0.744
Error	1579.941	63	25.078		

Table 30 – ANOVA Table for 6% Ethanol or Water Self-Administration in the 2BC Task

Source	Sum of Squares	df	Mean Square	F	Sig.
Housing	1864.196	1	1864.196	27.266	0.000
Stress	43.79405	1	43.794	0.641	0.427
Ethanol	1.649318	1	1.649	0.024	0.877
Housing * Stress	32.55319	1	32.553	0.476	0.493
Housing * Ethanol	0.042015	1	0.042	0.001	0.980
Stress * Ethanol	132.2501	1	132.250	1.934	0.169
Housing * Stress * Ethanol	38.35632	1	38.356	0.561	0.457
Error	4238.952	62	68.370		

Table 31 – ANOVA Table for 12% Ethanol or Water Self-Administration in the 2BC Task

Source	Sum of Squares	df	Mean Square	F	Sig.
Housing	281.675	1	281.675	7.070	0.010
Stress	104.377	1	104.377	2.620	0.110
Ethanol	31.668	1	31.668	0.795	0.376
Housing * Stress	389.810	1	389.810	9.784	0.003
Housing * Ethanol	134.671	1	134.671	3.380	0.071
Stress * Ethanol	184.929	1	184.929	4.641	0.035
Housing * Stress * Ethanol	0.010	1	0.010	0.000	0.987
Error	2549.946	64	39.843		

Table 32 – Repeated-Measures ANOVA Table for Water Consumption, Within-Subject Effects

Source	Sum of Squares		df	Mean Square	F	Sig.
phase	Sphericity Assumed	5853.577	2	2926.788	36.329	0.000
	Greenhouse-Geisser	5853.577	1.685	3474.187	36.329	0.000
phase * Housing	Sphericity Assumed	804.820	2	402.410	4.995	0.008
	Greenhouse-Geisser	804.820	1.685	477.673	4.995	0.012
phase * Stress	Sphericity Assumed	805.290	2	402.645	4.998	0.008
	Greenhouse-Geisser	805.290	1.685	477.952	4.998	0.012
phase * Ethanol	Sphericity Assumed	601.414	2	300.707	3.733	0.027
	Greenhouse-Geisser	601.414	1.685	356.948	3.733	0.034
phase * Housing * Stress	Sphericity Assumed	337.233	2	168.616	2.093	0.128
	Greenhouse-Geisser	337.233	1.685	200.153	2.093	0.137
phase * Housing * Ethanol	Sphericity Assumed	406.696	2	203.348	2.524	0.085
	Greenhouse-Geisser	406.696	1.685	241.380	2.524	0.094
phase * Stress * Ethanol	Sphericity Assumed	33.387	2	16.693	0.207	0.813
	Greenhouse-Geisser	33.387	1.685	19.816	0.207	0.775
phase * Housing * Stress * Ethanol	Sphericity Assumed	76.705	2	38.353	0.476	0.622
	Greenhouse-Geisser	76.705	1.685	45.526	0.476	0.590
Error	Sphericity Assumed	9345.432	116	80.564		
	Greenhouse-Geisser	9345.432	97.723	95.632		

Table 33 – Repeated-Measures ANOVA Table for Water Consumption, Between-Subjects Effects

Source	Sum of Squares	df	Mean Square	F	Sig.
Stress	103.679	1	103.679	0.823	0.368
Ethanol	54.083	1	54.083	0.429	0.515
Housing * Stress	1561.275	1	1561.275	12.395	0.001
Housing * Ethanol	4.463	1	4.463	0.035	0.851
Stress * Ethanol	34.130	1	34.130	0.271	0.605
Housing * Stress * Ethanol	65.441	1	65.441	0.520	0.474
Error	7305.972	58	125.965		

Table 34 – ANOVA Table for Water Consumption during Baseline Phase

Source	Sum of Squares	df	Mean Square	F	Sig.
Housing	86.766	1	86.766	0.951	0.333
Stress	25.619	1	25.619	0.281	0.598
Ethanol	304.808	1	304.808	3.340	0.073
Housing * Stress	126.519	1	126.519	1.386	0.244
Housing * Ethanol	42.325	1	42.325	0.464	0.498
Stress * Ethanol	33.827	1	33.827	0.371	0.545
Housing * Stress * Ethanol	13.990	1	13.990	0.153	0.697
Error	5567.022	61	91.263		

Table 35 – ANOVA Table for Water Consumption during the 2BC Phase

Source	Sum of Squares	df	Mean Square	F	Sig.
Housing	2125.889	1	2125.889	18.742	0.000
Stress	1030.684	1	1030.684	9.087	0.004
Ethanol	173.995	1	173.995	1.534	0.220
Housing * Stress	871.384	1	871.384	7.682	0.007
Housing * Ethanol	437.055	1	437.055	3.853	0.054
Stress * Ethanol	83.884	1	83.884	0.740	0.393
Housing * Stress * Ethanol	221.903	1	221.903	1.956	0.167
Error	7032.506	62	113.428		

Table 36 – ANOVA Table for Water Consumption during the Operant LSA Phase

Source	Sum of Squares	df	Mean Square	F	Sig.
Housing	513.481	1	513.481	5.711	0.020
Stress	248.470	1	248.470	2.764	0.101
Ethanol	219.505	1	219.505	2.441	0.123
Housing * Stress	740.591	1	740.591	8.237	0.006
Housing * Ethanol	59.522	1	59.522	0.662	0.419
Stress * Ethanol	3.162	1	3.162	0.035	0.852
Housing * Stress * Ethanol	4.156	1	4.156	0.046	0.830
Error	5664.302	63	89.910		

Table 37 – Repeated-Measures ANOVA Table for Food Consumption, Within-Subject Effects (Sphericity Assumed)

Source	Sum of Squares	df	Mean Square	F	Sig.
phase	966.921	2	483.460	71.967	0.000
phase * Housing	8.964	2	4.482	0.667	0.515
phase * Stress	15.077	2	7.538	1.122	0.329
phase * Ethanol	51.056	2	25.528	3.800	0.025
phase * Housing * Stress	9.832	2	4.916	0.732	0.483
phase * Housing * Ethanol	38.938	2	19.469	2.898	0.059
phase * Stress * Ethanol	8.524	2	4.262	0.634	0.532
phase * Housing * Stress * Ethanol	112.186	2	56.093	8.350	0.000
Error	819.575	122	6.718		

Table 38 – Repeated-Measures ANOVA Table for Food Consumption, Between-Subjects Effects

Source	Sum of Squares	df	Mean Square	F	Sig.
Housing	43.432	1	43.432	4.473	0.039
Stress	9.179	1	9.179	0.945	0.335
Ethanol	25.418	1	25.418	2.618	0.111
Housing * Stress	82.856	1	82.856	8.533	0.005
Housing * Ethanol	8.016	1	8.016	0.825	0.367
Stress * Ethanol	21.698	1	21.698	2.235	0.140
Housing * Stress * Ethanol	1.728	1	1.728	0.178	0.675
Error	592.333	61	9.710		

Table 39 – ANOVA Table for Food Consumption during Baseline Phase

Source	Sum of Squares	df	Mean Square	F	Sig.
Housing	11.568	1	11.568	1.633	0.206
Stress	0.058	1	0.058	0.008	0.928
Ethanol	81.920	1	81.920	11.562	0.001
Housing * Stress	14.906	1	14.906	2.104	0.152
Housing * Ethanol	1.445	1	1.445	0.204	0.653
Stress * Ethanol	3.067	1	3.067	0.433	0.513
Housing * Stress * Ethanol	59.878	1	59.878	8.451	0.005
Error	453.452	64	7.085		

Table 40 – ANOVA Table for Food Consumption during the 2BC Phase

Source	Sum of Squares	df	Mean Square	F	Sig.
Housing	26.113	1	26.113	3.130	0.082
Stress	0.288	1	0.288	0.035	0.853
Ethanol	0.255	1	0.255	0.031	0.862
Housing * Stress	43.218	1	43.218	5.181	0.026
Housing * Ethanol	1.493	1	1.493	0.179	0.674
Stress * Ethanol	19.476	1	19.476	2.335	0.132
Housing * Stress * Ethanol	5.631	1	5.631	0.675	0.414
Error	517.180	62	8.342		

Table 41 – ANOVA Table for Food Consumption during the Operant LSA Phase

Source	Sum of Squares	df	Mean Square	F	Sig.
Housing	0.297	1	0.297	0.034	0.853
Stress	33.947	1	33.947	3.933	0.052
Ethanol	0.628	1	0.628	0.073	0.788
Housing * Stress	46.646	1	46.646	5.404	0.023
Housing * Ethanol	38.651	1	38.651	4.478	0.038
Stress * Ethanol	3.806	1	3.806	0.441	0.509
Housing * Stress * Ethanol	56.899	1	56.899	6.592	0.013
Error	543.756	63	8.631		

Table 42 – ANOVA Table for Number of Licks of 3% Ethanol in Operant Liquid Self-Administration Paradigm

Source	Sum of Squares	df	Mean Square	F	Sig.
Stress	10.640	1	10.640	0.033	0.856
Housing	2469.143	1	2469.143	7.733	0.009
Stress * Housing	131.157	1	131.157	0.411	0.526
Error	10218.222	32	319.319		

Table 43 – ANOVA Table for Number of Lever Presses for 3% Ethanol in Operant Liquid Self-Administration Paradigm

Source	Sum of Squares	df	Mean Square	F	Sig.
Stress	2.377	1	2.377	0.312	0.580
Housing	35.007	1	35.007	4.595	0.040
Stress * Housing	28.002	1	28.002	3.676	0.064
Error	243.771	32	7.618		

Table 44 – ANOVA Table for Number of Lever Presses for Water in Operant Liquid Self-Administration Paradigm (3% Ethanol Phase)

Source	Sum of Squares	df	Mean Square	F	Sig.
Stress	4.587	1	4.587	0.124	0.727
Housing	91.617	1	91.617	2.481	0.125
Stress * Housing	16.040	1	16.040	0.434	0.515
Error	1181.640	32	36.926		

Table 45 – ANOVA Table for Number of Licks of 6% Ethanol in Operant Liquid Self-Administration Paradigm

Source	Sum of Squares	df	Mean Square	F	Sig.
Stress	4.474	1	4.474	0.032	0.860
Housing	296.195	1	296.195	2.108	0.156
Stress * Housing	461.738	1	461.738	3.286	0.079
Error	4496.347	32	140.511		

Table 46 – ANOVA Table for Number of Lever Presses for 6% Ethanol in Operant Liquid Self-Administration Paradigm

Source	Sum of Squares	df	Mean Square	F	Sig.
Stress	0.580	1	0.580	0.087	0.770
Housing	31.574	1	31.574	4.739	0.037
Stress * Housing	17.961	1	17.961	2.696	0.110
Error	213.211	32	6.663		

Table 47 – ANOVA Table for Number of Lever Presses for Water in Operant Liquid Self-Administration Paradigm (6% Ethanol Phase)

Source	Sum of Squares	df	Mean Square	F	Sig.
Stress	18.376	1	18.376	0.881	0.355
Housing	5.214	1	5.214	0.250	0.621
Stress * Housing	83.235	1	83.235	3.989	0.054
Error	667.668	32	20.865		

Table 48 – ANOVA Table for Number of Licks of 12% Ethanol in Operant Liquid Self-Administration Paradigm

Source	Sum of Squares	df	Mean Square	F	Sig.
Stress	20.379	1	20.379	0.098	0.756
Housing	615.040	1	615.040	2.971	0.094
Stress * Housing	445.813	1	445.813	2.153	0.152
Error	6624.848	32	207.026		

Table 49 – ANOVA Table for Number of Lever Presses for 12% Ethanol in Operant Liquid Self-Administration Paradigm

Source	Sum of Squares	df	Mean Square	F	Sig.
Stress	3.738	1	3.738	2.977	0.094
Housing	12.217	1	12.217	9.731	0.004
Stress * Housing	6.370	1	6.370	5.074	0.031
Error	40.173	32	1.255		

Table 50 – ANOVA Table for Number of Lever Presses for Water in Operant Liquid Self-Administration Paradigm (12% Ethanol Phase)

Source	Sum of Squares	df	Mean Square	F	Sig.
Stress	36.401	1	36.401	1.968	0.170
Housing	7.182	1	7.182	0.388	0.538
Stress * Housing	17.445	1	17.445	0.943	0.339
Error	592.037	32	18.501		

Table 51a – MANOVA Tables for Open Field Activity Parameters during Baseline

Multivariate Test					
Effect	***V* Value**	**F**	**df**	**Error df**	**Sig.**
Housing	0.433	11.628	4	61	0.000
Stress	0.022	0.336	4	61	0.852
Ethanol	0.034	0.542	4	61	0.705
Housing * Stress	0.150	2.686	4	61	0.040
Housing * Ethanol	0.031	0.487	4	61	0.746
Stress * Ethanol	0.038	0.602	4	61	0.663
Housing * Stress * Ethanol	0.016	0.245	4	61	0.911

Table 51b – MANOVA Tables for Open Field Activity Parameters during Baseline

Test of Between-Subjects Effects						
Source	**Dependent Variable**	**Sum of Squares**	**df**	**Mean Square**	**F**	**Sig.**
Housing	Horiz. Activity	438400725	1	438400725	44.596	0.000
	Total Dist.	132850433	1	132850433	43.170	0.000
	Vert. Activity	2144520	1	2144520	13.213	0.001
	Center Time	354215	1	354215	8.553	0.005
Stress	Horiz. Activity	2070273	1	2070273	0.211	0.648
	Total Dist.	539414	1	539414	0.175	0.677
	Vert. Activity	29768	1	29768	0.183	0.670
	Center Time	2197	1	2197	0.053	0.819
Ethanol	Horiz. Activity	9501167	1	9501167	0.966	0.329
	Total Dist.	2674985	1	2674985	0.869	0.355
	Vert. Activity	8493	1	8493	0.052	0.820
	Center Time	44616	1	44616	1.077	0.303
Housing * Stress	Horiz. Activity	61636454	1	61636454	6.270	0.015
	Total Dist.	5782267	1	5782267	1.879	0.175
	Vert. Activity	593687	1	593687	3.658	0.060
	Center Time	125726	1	125726	3.036	0.086
Housing * Ethanol	Horiz. Activity	12130275	1	12130275	1.234	0.271
	Total Dist.	1585981	1	1585981	0.515	0.475
	Vert. Activity	141512	1	141512	0.872	0.354
	Center Time	43390	1	43390	1.048	0.310
Stress * Ethanol	Horiz. Activity	1117762	1	1117762	0.114	0.737
	Total Dist.	416480	1	416480	0.135	0.714
	Vert. Activity	5513	1	5513	0.034	0.854
	Center Time	1	1	1	0.000	0.996
Housing * Stress * Ethanol	Horiz. Activity	118341	1	118341	0.012	0.913
	Total Dist.	624	1	624	0.000	0.989
	Vert. Activity	59168	1	59168	0.365	0.548
	Center Time	27048	1	27048	0.653	0.422
Error	Horiz. Activity	629156159	64	9830565		
	Total Dist.	196954086	64	3077408		
	Vert. Activity	10387137	64	162299		
	Center Time	2650493	64	41414		

Table 52 – ANOVA Table for Horizontal Activity during the Baseline Phase

Source	Sum of Squares	df	Mean Square	F	Sig.
Alcohol	2957630.769	1	2957630.769	0.107	0.745
Stress	27277011.692	1	27277011.692	0.986	0.325
Housing	477264789.926	1	477264789.926	17.261	0.000
Alcohol * Stress	89607729.641	1	89607729.641	3.241	0.077
Alcohol * Housing	4471303.293	1	4471303.293	0.162	0.689
Stress * Housing	138730388.103	1	138730388.103	5.017	0.029
Alcohol * Stress * Housing	2749208.251	1	2749208.251	0.099	0.754
Error	1548416663.778	56	27650297.567		

Table 53 – ANOVA Table for Total Distance Traveled during Baseline Phase

Source	Sum of Squares	df	Mean Square	F	Sig.
Alcohol	4613384.160	1	4613384.160	0.491	0.486
Stress	10382436.058	1	10382436.058	1.106	0.297
Housing	190800484.776	1	190800484.776	20.323	0.000
Alcohol * Stress	28241853.368	1	28241853.368	3.008	0.088
Alcohol * Housing	210687.750	1	210687.750	0.022	0.881
Stress * Housing	39277491.853	1	39277491.853	4.184	0.046
Alcohol * Stress * Housing	4050870.531	1	4050870.531	0.431	0.514
Error	525758414.972	56	9388543.125		

Table 54 – ANOVA Table for Vertical Activity during Baseline Phase

Source	Sum of Squares	df	Mean Square	F	Sig.
Alcohol	60127.738	1	60127.738	0.128	0.721
Stress	119335.567	1	119335.567	0.255	0.616
Housing	4907641.071	1	4907641.071	10.483	0.002
Alcohol * Stress	380064.103	1	380064.103	0.812	0.371
Alcohol * Housing	128426.769	1	128426.769	0.274	0.603
Stress * Housing	1569614.769	1	1569614.769	3.353	0.072
Alcohol * Stress * Housing	7384.900	1	7384.900	0.016	0.901
Error	26217404.944	56	468167.945		

Table 55 – ANOVA Table for Center Time during Baseline Phase

Source	Sum of Squares	df	Mean Square	F	Sig.
Alcohol	192121.027	1	192121.027	0.859	0.358
Stress	219637.579	1	219637.579	0.982	0.326
Housing	503009.480	1	503009.480	2.249	0.139
Alcohol * Stress	236852.389	1	236852.389	1.059	0.308
Alcohol * Housing	548576.393	1	548576.393	2.453	0.123
Stress * Housing	254221.194	1	254221.194	1.137	0.291
Alcohol * Stress * Housing	102340.293	1	102340.293	0.458	0.502
Error	12525998.539	56	223678.545		
Total	86089144.870	64			

Table 56a – MANOVA Table for Open Field Activity Parameters during the 2BC Phase

Multivariate Test					
Effect	***V* Value**	**F**	**df**	**Error df**	**Sig.**
Alcohol	0.048	0.675	4	53	0.613
Stress	0.030	0.406	4	53	0.803
Housing	0.270	4.910	4	53	0.002
Alcohol * Stress	0.066	0.942	4	53	0.447
Alcohol * Housing	0.077	1.106	4	53	0.364
Stress * Housing	0.084	1.222	4	53	0.313
Alcohol * Stress * Housing	0.045	0.625	4	53	0.647

Table 56b – MANOVA Table for Open Field Activity Parameters during the 2BC Phase

Test of Between-Subjects Effects						
Source	**Dependent Variable**	**Sum of Squares**	**df**	**Mean Square**	**F**	**Sig.**
Ethanol	Horiz. Activity	2957631	1	2957631	0.107	0.745
	Total Dist.	4613384	1	4613384	0.491	0.486
	Vert. Activity	60128	1	60128	0.128	0.721
	Center Time	192121	1	192121	0.859	0.358
Stress	Horiz. Activity	27277012	1	27277012	0.986	0.325
	Total Dist.	10382436	1	10382436	1.106	0.297
	Vert. Activity	119336	1	119336	0.255	0.616
	Center Time	219638	1	219638	0.982	0.326
Housing	Horiz. Activity	477264790	1	477264790	17.261	0.000
	Total Dist.	190800485	1	190800485	20.323	0.000
	Vert. Activity	4907641	1	4907641	10.483	0.002
	Center Time	503009	1	503009	2.249	0.139
Ethanol * Stress	Horiz. Activity	89607730	1	89607730	3.241	0.077
	Total Dist.	28241853	1	28241853	3.008	0.088
	Vert. Activity	380064	1	380064	0.812	0.371
	Center Time	236852	1	236852	1.059	0.308
Housing * Ethanol	Horiz. Activity	4471303	1	4471303	0.162	0.689
	Total Dist.	210688	1	210688	0.022	0.881
	Vert. Activity	128427	1	128427	0.274	0.603
	Center Time	548576	1	548576	2.453	0.123
Stress * Housing	Horiz. Activity	138730388	1	138730388	5.017	0.029
	Total Dist.	39277492	1	39277492	4.184	0.046
	Vert. Activity	1569615	1	1569615	3.353	0.072
	Center Time	254221	1	254221	1.137	0.291
Housing * Stress * Ethanol	Horiz. Activity	2749208	1	2749208	0.099	0.754
	Total Dist.	4050871	1	4050871	0.431	0.514
	Vert. Activity	7385	1	7385	0.016	0.901
	Center Time	102340	1	102340	0.458	0.502
Error	Horiz. Activity	1548416664	56	27650298		
	Total Dist.	525758415	56	9388543		
	Vert. Activity	26217405	56	468168		
	Center Time	12525999	56	223679		

Table 57 – ANOVA Table for Horizontal Activity during the 2BC Phase

Source	Sum of Squares	df	Mean Square	F	Sig.
Housing	438400725.347	1	438400725.347	44.596	0.000
Stress	2070273.347	1	2070273.347	0.211	0.648
Ethanol	9501167.014	1	9501167.014	0.966	0.329
Housing * Stress	61636454.014	1	61636454.014	6.270	0.015
Housing * Ethanol	12130275.125	1	12130275.125	1.234	0.271
Stress * Ethanol	1117761.681	1	1117761.681	0.114	0.737
Housing * Stress * Ethanol	118341.125	1	118341.125	0.012	0.913
Error	629156158.667	64	9830564.979		

Table 58 – ANOVA Table for Total Distance Traveled during the 2BC Phase

Source	Sum of Squares	df	Mean Square	F	Sig.
Housing	132850433.389	1	132850433.389	43.170	0.000
Stress	539414.222	1	539414.222	0.175	0.677
Ethanol	2674984.500	1	2674984.500	0.869	0.355
Housing * Stress	5782266.889	1	5782266.889	1.879	0.175
Housing * Ethanol	1585980.500	1	1585980.500	0.515	0.475
Stress * Ethanol	416480.222	1	416480.222	0.135	0.714
Housing * Stress * Ethanol	624.222	1	624.222	0.000	0.989
Error	196954085.556	64	3077407.587		

Table 59 – ANOVA Table for Vertical Activity during the 2BC Phase

Source	Sum of Squares	df	Mean Square	F	Sig.
Housing	2144520.500	1	2144520.500	13.213	0.001
Stress	29768.000	1	29768.000	0.183	0.670
Ethanol	8493.389	1	8493.389	0.052	0.820
Housing * Stress	593686.722	1	593686.722	3.658	0.060
Housing * Ethanol	141512.000	1	141512.000	0.872	0.354
Stress * Ethanol	5512.500	1	5512.500	0.034	0.854
Housing * Stress * Ethanol	59168.000	1	59168.000	0.365	0.548
Error	10387136.889	64	162299.014		

Table 60 – ANOVA Table for Center Time during the 2BC Phase

Source	Sum of Squares	df	Mean Square	F	Sig.
Housing	354215.417	1	354215.417	8.553	0.005
Stress	2196.740	1	2196.740	0.053	0.819
Ethanol	44615.823	1	44615.823	1.077	0.303
Housing * Stress	125726.051	1	125726.051	3.036	0.086
Housing * Ethanol	43389.670	1	43389.670	1.048	0.310
Stress * Ethanol	1.201	1	1.201	0.000	0.996
Housing * Stress * Ethanol	27047.503	1	27047.503	0.653	0.422
Error	2650492.982	64	41413.953		

Table 61a – MANOVA Table for Open Field Activity Parameters during the Operant LSA Phase

Multivariate Test					
Effect	***V* Value**	**F**	**df**	**Error df**	**Sig.**
Alcohol	0.054	0.868	4	61	0.488
Stress	0.088	1.466	4	61	0.223
Housing	0.284	6.046	4	61	0.000
Alcohol * Stress	0.065	1.058	4	61	0.385
Alcohol * Housing	0.081	1.336	4	61	0.267
Stress * Housing	0.101	1.722	4	61	0.157
Alcohol * Stress * Housing	0.029	0.457	4	61	0.767

Table 61b – MANOVA Table for Open Field Activity Parameters during the Operant LSA Phase

Test of Between-Subjects Effects						
Source	**Dependent Variable**	**Sum of Squares**	**df**	**Mean Square**	**F**	**Sig.**
Ethanol	Horiz. Activity	3362	1	3362	0.000	0.986
	Total Dist.	1710942	1	1710942	0.380	0.540
	Vert. Activity	2990	1	2990	0.006	0.940
	Center Time	2640	1	2640	0.013	0.910
Stress	Horiz. Activity	7579022	1	7579022	0.692	0.409
	Total Dist.	756655	1	756655	0.168	0.683
	Vert. Activity	801378	1	801378	1.547	0.218
	Center Time	196	1	196	0.001	0.976
Housing	Horiz. Activity	124741013	1	124741013	11.393	0.001
	Total Dist.	64141026	1	64141026	14.238	0.000
	Vert. Activity	6038971	1	6038971	11.656	0.001
	Center Time	1386335	1	1386335	6.688	0.012
Ethanol * Stress	Horiz. Activity	4699156	1	4699156	0.429	0.515
	Total Dist.	3081	1	3081	0.001	0.979
	Vert. Activity	297735	1	297735	0.575	0.451
	Center Time	31937	1	31937	0.154	0.696
Housing * Ethanol	Horiz. Activity	1789832	1	1789832	0.163	0.687
	Total Dist.	363662	1	363662	0.081	0.777
	Vert. Activity	445253	1	445253	0.859	0.357
	Center Time	347500	1	347500	1.676	0.200
Stress * Housing	Horiz. Activity	25675778	1	25675778	2.345	0.131
	Total Dist.	13906659	1	13906659	3.087	0.084
	Vert. Activity	3202981	1	3202981	6.182	0.016
	Center Time	189892	1	189892	0.916	0.342
Housing * Stress * Ethanol	Horiz. Activity	90596	1	90596	0.008	0.928
	Total Dist.	324952	1	324952	0.072	0.789
	Vert. Activity	8624	1	8624	0.017	0.898
	Center Time	272568	1	272568	1.315	0.256
Error	Horiz. Activity	700723147	64	10948799		
	Total Dist.	288321727	64	4505027		
	Vert. Activity	33157380	64	518084		
	Center Time	13266344	64	207287		

Table 62 – ANOVA Table for Horizontal Activity during the Operant LSA Phase

Source	Sum of Squares	df	Mean Square	F	Sig.
Alcohol	3362.000	1	3362.000	0.000	0.986
Stress	7579022.222	1	7579022.222	0.692	0.409
Housing	124741012.500	1	124741012.500	11.393	0.001
Alcohol * Stress	4699156.056	1	4699156.056	0.429	0.515
Alcohol * Housing	1789832.000	1	1789832.000	0.163	0.687
Stress * Housing	25675778.000	1	25675778.000	2.345	0.131
Alcohol * Stress * Housing	90596.056	1	90596.056	0.008	0.928
Error	700723147.111	64	10948799.174		

Table 63 – ANOVA Table for Total Distance Traveled during the Operant LSA Phase

Source	Sum of Squares	df	Mean Square	F	Sig.
Alcohol	1710941.681	1	1710941.681	0.380	0.540
Stress	756655.014	1	756655.014	0.168	0.683
Housing	64141025.681	1	64141025.681	14.238	0.000
Alcohol * Stress	3081.125	1	3081.125	0.001	0.979
Alcohol * Housing	363662.347	1	363662.347	0.081	0.777
Stress * Housing	13906659.014	1	13906659.014	3.087	0.084
Alcohol * Stress * Housing	324952.347	1	324952.347	0.072	0.789
Error	288321726.667	64	4505026.979		

Table 64 – ANOVA Table for Vertical Activity during the Operant LSA Phase

Source	Sum of Squares	df	Mean Square	F	Sig.
Alcohol	2990.222	1	2990.222	0.006	0.940
Stress	801378.000	1	801378.000	1.547	0.218
Housing	6038970.889	1	6038970.889	11.656	0.001
Alcohol * Stress	297734.722	1	297734.722	0.575	0.451
Alcohol * Housing	445253.389	1	445253.389	0.859	0.357
Stress * Housing	3202980.500	1	3202980.500	6.182	0.016
Alcohol * Stress * Housing	8624.222	1	8624.222	0.017	0.898
Error	33157379.556	64	518084.056		

Table 65 – ANOVA Table for Center Time during Operant LSA Phase

Source	Sum of Squares	df	Mean Square	F	Sig.
Alcohol	2640.222	1	2640.222	0.013	0.910
Stress	196.020	1	196.020	0.001	0.976
Housing	1386334.509	1	1386334.509	6.688	0.012
Alcohol * Stress	31937.069	1	31937.069	0.154	0.696
Alcohol * Housing	347500.056	1	347500.056	1.676	0.200
Stress * Housing	189892.302	1	189892.302	0.916	0.342
Alcohol * Stress * Housing	272568.056	1	272568.056	1.315	0.256
Error	13266344.378	64	207286.631		

Table 66a – MANOVA Table for Forced Swim Test Parameters during the 2BC Phase

Multivariate Test					
Effect	***V* Value**	**F**	**df**	**Error df**	**Sig.**
Ethanol	0.003	0.085	2	60	0.918
Stress	0.072	2.316	2	60	0.107
Housing	0.023	0.710	2	60	0.496
Ethanol * Stress	0.087	2.863	2	60	0.065
Ethanol * Housing	0.031	0.951	2	60	0.392
Stress * Housing	0.031	0.963	2	60	0.388
Ethanol * Stress * Housing	0.039	1.219	2	60	0.303

Table 66b – MANOVA Table for Forced Swim Test Parameters during the 2BC Phase

Source	Dependent Variable	Sum of Squares	df	Mean Square	F	Sig.
Ethanol	Time Spent Immobile	159.360	1	159.360	0.430	0.514
	# Immobile Episodes	13.462	1	13.462	0.321	0.573
Stress	Time Spent Immobile	276.719	1	276.719	0.747	0.391
	# Immobile Episodes	29.728	1	29.728	0.709	0.403
Housing	Time Spent Immobile	231.645	1	231.645	0.625	0.432
	# Immobile Episodes	54.925	1	54.925	1.310	0.257
Ethanol * Stress	Time Spent Immobile	3138.310	1	3138.310	8.473	0.005
	# Immobile Episodes	426.617	1	426.617	10.177	0.002
Ethanol * Housing	Time Spent Immobile	1545.256	1	1545.256	4.172	0.045
	# Immobile Episodes	151.848	1	151.848	3.622	0.062
Stress * Housing	Time Spent Immobile	529.375	1	529.375	1.429	0.236
	# Immobile Episodes	30.693	1	30.693	0.732	0.395
Ethanol * Stress * Housing	Time Spent Immobile	1152.863	1	1152.863	3.113	0.083
	# Immobile Episodes	140.539	1	140.539	3.353	0.072
Error	Time Spent Immobile	23333.271	63	370.369		
	# Immobile Episodes	2640.986	63	41.920		

Table 67 – ANOVA Table for Time Spent Immobile during the 2BC Phase

Source	Sum of Squares	df	Mean Square	F	Sig.
Ethanol	159.360	1	159.360	0.430	0.514
Stress	276.719	1	276.719	0.747	0.391
Housing	231.645	1	231.645	0.625	0.432
Ethanol * Stress	3138.310	1	3138.310	8.473	0.005
Ethanol * Housing	1545.256	1	1545.256	4.172	0.045
Stress * Housing	529.375	1	529.375	1.429	0.236
Ethanol * Stress * Housing	1152.863	1	1152.863	3.113	0.083
Error	23333.271	63	370.369		

Table 68 – ANOVA Table for Number of Immobile Episodes During the 2BC Phase

Source	Sum of Squares	df	Mean Square	F	Sig.
Ethanol	13.462	1	13.462	0.321	0.573
Stress	29.728	1	29.728	0.709	0.403
Housing	54.925	1	54.925	1.310	0.257
Ethanol * Stress	426.617	1	426.617	10.177	0.002
Ethanol * Housing	151.848	1	151.848	3.622	0.062
Stress * Housing	30.693	1	30.693	0.732	0.395
Ethanol * Stress * Housing	140.539	1	140.539	3.353	0.072
Error	2640.986	63	41.920		

Table 69a – MANOVA Table for Forced Swim Test Parameters during the Operant LSA Phase

Multivariate Test					
Effect	***V* Value**	**F**	**df**	**Error df**	**Sig.**
Ethanol	0.019	0.569	2	60	0.569
Stress	0.027	0.821	2	60	0.445
Housing	0.041	1.270	2	60	0.288
Ethanol * Stress	0.066	2.104	2	60	0.131
Ethanol * Housing	0.006	0.186	2	60	0.831
Stress * Housing	0.050	1.573	2	60	0.216
Ethanol * Stress * Housing	0.067	2.170	2	60	0.123

Table 69b – MANOVA Table for Forced Swim Test Parameters during the Operant LSA Phase

Test of Between-Subjects Effects						
Source	**Dependent Variable**	**Sum of Squares**	**df**	**Mean Square**	**F**	**Sig.**
Ethanol	Time Spent Immobile	84.062	1	84.062	0.193	0.662
	# Immobile Episodes	16.485	1	16.485	0.310	0.580
Stress	Time Spent Immobile	319.872	1	319.872	0.735	0.394
	# Immobile Episodes	58.230	1	58.230	1.095	0.299
Housing	Time Spent Immobile	2379.185	1	2379.185	5.471	0.023
	# Immobile Episodes	399.367	1	399.367	7.513	0.008
Ethanol * Stress	Time Spent Immobile	3718.267	1	3718.267	8.550	0.005
	# Immobile Episodes	365.585	1	365.585	6.878	0.011
Ethanol * Housing	Time Spent Immobile	1040.133	1	1040.133	2.392	0.127
	# Immobile Episodes	119.105	1	119.105	2.241	0.140
Stress * Housing	Time Spent Immobile	373.000	1	373.000	0.858	0.358
	# Immobile Episodes	17.434	1	17.434	0.328	0.569
Ethanol * Stress * Housing	Time Spent Immobile	1732.802	1	1732.802	3.984	0.050
	# Immobile Episodes	191.045	1	191.045	3.594	0.063
Error	Time Spent Immobile	26529.466	61	434.909		
	# Immobile Episodes	3242.472	61	53.155		

Table 70 – ANOVA Table for Total Time Spent Immobile during the Operant LSA Phase

Source	Sum of Squares	df	Mean Square	F	Sig.
Ethanol	176.993	1	176.993	0.397	0.531
Stress	194.708	1	194.708	0.437	0.511
Housing	2032.104	1	2032.104	4.561	0.037
Ethanol * Stress	4290.770	1	4290.770	9.631	0.003
Ethanol * Housing	808.170	1	808.170	1.814	0.183
Stress * Housing	236.939	1	236.939	0.532	0.469
Ethanol * Stress * Housing	1434.512	1	1434.512	3.220	0.078
Error	27620.690	62	445.495		

Table 71 – ANOVA Table for Number of Immobile Episodes during the Operant LSA Phase

Source	Sum of Squares	df	Mean Square	F	Sig.
Ethanol	0.694	1	0.694	0.011	0.918
Stress	65.344	1	65.344	1.018	0.317
Housing	235.128	1	235.128	3.663	0.060
Ethanol * Stress	390.000	1	390.000	6.075	0.016
Ethanol * Housing	257.139	1	257.139	4.006	0.050
Stress * Housing	20.934	1	20.934	0.326	0.570
Ethanol * Stress * Housing	187.600	1	187.600	2.922	0.092
Error	4044.208	63	64.194		

Table 72 – Correlation Matrix for Neurochemicals in the Nucleus Accumbens

Neurochemical		NE	DOPAC	5-HIAA	DA	HVA	5-HT
NE	Pearson Correlation	1	0.544	0.282	0.609	0.648	0.464
	Sig. (2-tailed)		0.000	0.020	0.000	0.000	0.000
DOPAC	Pearson Correlation	0.544	1	0.329	0.824	0.840	0.394
	Sig. (2-tailed)	0.000		0.005	0.000	0.000	0.001
5-HIAA	Pearson Correlation	0.282	0.329	1	0.233	0.293	0.503
	Sig. (2-tailed)	0.020	0.005		0.053	0.015	0.000
DA	Pearson Correlation	0.609	0.824	0.233	1	0.848	0.616
	Sig. (2-tailed)	0.000	0.000	0.053		0.000	0.000
HVA	Pearson Correlation	0.648	0.840	0.293	0.848	1	0.545
	Sig. (2-tailed)	0.000	0.000	0.015	0.000		0.000
5-HT	Pearson Correlation	0.464	0.394	0.503	0.616	0.545	1
	Sig. (2-tailed)	0.000	0.001	0.000	0.000	0.000	

Table 73a – MANOVA Table for Neurochemicals in the Nucleus Accumbens

Multivariate Test					
Effect	***V* Value**	**F**	**df**	**Error df**	**Sig.**
Ethanol	0.196	2.071	6	51	0.073
Stress	0.245	2.762	6	51	0.021
Housing	0.611	13.378	6	51	0.000
Ethanol * Stress	0.100	0.942	6	51	0.474
Ethanol * Housing	0.127	1.233	6	51	0.305
Stress * Housing	0.167	1.709	6	51	0.138
Ethanol * Stress * Housing	0.143	1.414	6	51	0.227

Table 73b – MANOVA Table for Neurochemicals in the Nucleus Accumbens

Test of Between-Subjects Effects						
Source	**Dependent Variable**	**Sum of Squares**	**df**	**Mean Square**	**F**	**Sig.**
Ethanol	NE	4.016	1	4.016	0.431	0.514
	DOPAC	291.245	1	291.245	2.217	0.142
	5-HIAA	10.959	1	10.959	4.889	0.031
	DA	5537.854	1	5537.854	2.036	0.159
	HVA	4.332	1	4.332	0.296	0.589
	5-HT	5.946	1	5.946	2.778	0.101
Stress	NE	79.380	1	79.380	8.525	0.005
	DOPAC	21.748	1	21.748	0.166	0.686
	5-HIAA	19.286	1	19.286	8.603	0.005
	DA	73.271	1	73.271	0.027	0.870
	HVA	19.462	1	19.462	1.329	0.254
	5-HT	4.871	1	4.871	2.276	0.137
Housing	NE	54.462	1	54.462	5.849	0.019
	DOPAC	394.935	1	394.935	3.006	0.088
	5-HIAA	84.630	1	84.630	37.753	0.000
	DA	13947.827	1	13947.827	5.129	0.027
	HVA	70.111	1	70.111	4.786	0.033
	5-HT	1.237	1	1.237	0.578	0.450
Ethanol * Stress	NE	2.054	1	2.054	0.221	0.640
	DOPAC	218.944	1	218.944	1.667	0.202
	5-HIAA	2.191	1	2.191	0.977	0.327
	DA	6050.528	1	6050.528	2.225	0.141
	HVA	25.342	1	25.342	1.730	0.194
	5-HT	2.172	1	2.172	1.015	0.318
Ethanol * Housing	NE	37.346	1	37.346	4.011	0.050
	DOPAC	57.196	1	57.196	0.435	0.512
	5-HIAA	0.007	1	0.007	0.003	0.954
	DA	68.581	1	68.581	0.025	0.874
	HVA	2.139	1	2.139	0.146	0.704
	5-HT	0.287	1	0.287	0.134	0.715
Stress * Housing	NE	69.865	1	69.865	7.503	0.008
	DOPAC	217.938	1	217.938	1.659	0.203
	5-HIAA	2.010	1	2.010	0.897	0.348
	DA	7721.055	1	7721.055	2.839	0.098
	HVA	71.119	1	71.119	4.855	0.032
	5-HT	1.521	1	1.521	0.711	0.403
Ethanol * Stress * Housing	NE	0.982	1	0.982	0.105	0.747
	DOPAC	67.549	1	67.549	0.514	0.476
	5-HIAA	8.424	1	8.424	3.758	0.058
	DA	364.028	1	364.028	0.134	0.716
	HVA	9.736	1	9.736	0.665	0.418
	5-HT	0.366	1	0.366	0.171	0.681
Error	NE	521.458	56	9.312		
	DOPAC	7356.704	56	131.370		
	5-HIAA	125.534	56	2.242		
	DA	152294.068	56	2719.537		
	HVA	820.343	56	14.649		
	5-HT	119.862	56	2.140		

Table 74 – ANOVA Table for DA in the Nucleus Accumbens

Source	Sum of Squares	df	Mean Square	F	Sig.
Ethanol	78.129	1	78.129	0.023	0.880
Stress	256.481	1	256.481	0.075	0.785
Housing	6077.825	1	6077.825	1.776	0.187
Ethanol * Stress	572.618	1	572.618	0.167	0.684
Ethanol * Housing	481.286	1	481.286	0.141	0.709
Stress * Housing	7929.896	1	7929.896	2.317	0.133
Ethanol * Stress * Housing	349.722	1	349.722	0.102	0.750
Error	218994.376	64	3421.787		

Table 75 – ANOVA Table for DOPAC in the Nucleus Accumbens

Source	Sum of Squares	df	Mean Square	F	Sig.
Ethanol	1.126	1	1.126	0.067	0.796
Stress	37.115	1	37.115	2.218	0.141
Housing	42.478	1	42.478	2.539	0.116
Ethanol * Stress	10.495	1	10.495	0.627	0.431
Ethanol * Housing	10.391	1	10.391	0.621	0.434
Stress * Housing	84.712	1	84.712	5.063	0.028
Ethanol * Stress * Housing	7.765	1	7.765	0.464	0.498
Error	1037.421	62	16.733		

Table 76 – ANOVA Table for NE in the Nucleus Accumbens

Source	Sum of Squares	df	Mean Square	F	Sig.
Ethanol	3.018	1	3.018	0.246	0.622
Stress	86.704	1	86.704	7.058	0.010
Housing	64.476	1	64.476	5.249	0.025
Ethanol * Stress	1.464	1	1.464	0.119	0.731
Ethanol * Housing	47.273	1	47.273	3.848	0.054
Stress * Housing	103.422	1	103.422	8.419	0.005
Ethanol * Stress * Housing	7.120	1	7.120	0.580	0.449
Error	749.337	61	12.284		

Table 77 – ANOVA Table for 5-HIAA in the Nucleus Accumbens

Source	Sum of Squares	df	Mean Square	F	Sig.
Ethanol	2.547	1	2.547	0.959	0.331
Stress	19.033	1	19.033	7.169	0.009
Housing	104.827	1	104.827	39.483	0.000
Ethanol * Stress	6.640	1	6.640	2.501	0.119
Ethanol * Housing	1.233	1	1.233	0.464	0.498
Stress * Housing	1.111	1	1.111	0.419	0.520
Ethanol * Stress * Housing	11.515	1	11.515	4.337	0.041
Error	164.610	62	2.655		

Table 78 – Correlation Matrix for Neurochemicals in the Ventral Tegmental Area

Neurochemical		NE	DOPAC	5-HIAA	DA	HVA	5-HT
NE	Pearson Correlation	1	0.689	0.233	0.763	0.638	0.423
	Sig. (2-tailed)		0.000	0.078	0.000	0.000	0.001
DOPAC	Pearson Correlation	0.689	1	0.247	0.914	0.891	0.262
	Sig. (2-tailed)	0.000		0.057	0.000	0.000	0.047
5-HIAA	Pearson Correlation	0.233	0.247	1	0.167	0.287	0.468
	Sig. (2-tailed)	0.078	0.057		0.202	0.028	0.000
DA	Pearson Correlation	0.763	0.914	0.167	1	0.794	0.349
	Sig. (2-tailed)	0.000	0.000	0.202		0.000	0.007
HVA	Pearson Correlation	0.638	0.891	0.287	0.794	1	0.257
	Sig. (2-tailed)	0.000	0.000	0.028	0.000		0.054
5-HT	Pearson Correlation	0.423	0.262	0.468	0.349	0.257	1
	Sig. (2-tailed)	0.001	0.047	0.000	0.007	0.054	

Table 79a – MANOVA Table for Neurochemicals in the Ventral Tegmental Area

Multivariate Test					
Effect	***V* Value**	**F**	**df**	**Error df**	**Sig.**
Ethanol	0.133	1.048	6	41	0.409
Stress	0.083	0.618	6	41	0.715
Housing	0.162	1.326	6	41	0.268
Ethanol * Stress	0.090	0.675	6	41	0.671
Ethanol * Housing	0.130	1.026	6	41	0.423
Stress * Housing	0.146	1.165	6	41	0.343
Ethanol * Stress * Housing	0.320	3.213	6	41	0.011

Table 79b – MANOVA Table for Neurochemicals in the VTA

Test of Between-Subjects Effects						
Source	**Dependent Variable**	**Sum of Squares**	**df**	**Mean Square**	**F**	**Sig.**
Ethanol	NE	27.165	1	27.165	3.423	0.071
	DOPAC	9.684	1	9.684	2.101	0.154
	5-HIAA	0.015	1	0.015	0.016	0.900
	DA	363.349	1	363.349	3.784	0.058
	HVA	6.246	1	6.246	3.421	0.071
	5-HT	0.322	1	0.322	0.475	0.494
Stress	NE	10.908	1	10.908	1.374	0.247
	DOPAC	0.795	1	0.795	0.172	0.680
	5-HIAA	0.027	1	0.027	0.029	0.865
	DA	102.310	1	102.310	1.065	0.307
	HVA	0.049	1	0.049	0.027	0.871
	5-HT	0.637	1	0.637	0.940	0.337
Housing	NE	6.448	1	6.448	0.812	0.372
	DOPAC	6.104	1	6.104	1.324	0.256
	5-HIAA	0.149	1	0.149	0.161	0.690
	DA	39.562	1	39.562	0.412	0.524
	HVA	1.837	1	1.837	1.006	0.321
	5-HT	0.006	1	0.006	0.008	0.927
Ethanol * Stress	NE	0.407	1	0.407	0.051	0.822
	DOPAC	0.051	1	0.051	0.011	0.917
	5-HIAA	0.349	1	0.349	0.380	0.541
	DA	24.398	1	24.398	0.254	0.617
	HVA	0.344	1	0.344	0.188	0.666
	5-HT	0.473	1	0.473	0.698	0.408
Ethanol * Housing	NE	0.070	1	0.070	0.009	0.926
	DOPAC	0.279	1	0.279	0.061	0.807
	5-HIAA	3.361	1	3.361	3.652	0.062
	DA	15.639	1	15.639	0.163	0.688
	HVA	0.805	1	0.805	0.441	0.510
	5-HT	0.769	1	0.769	1.136	0.292
Stress * Housing	NE	19.923	1	19.923	2.510	0.120
	DOPAC	5.925	1	5.925	1.285	0.263
	5-HIAA	0.768	1	0.768	0.834	0.366
	DA	132.848	1	132.848	1.383	0.246
	HVA	1.757	1	1.757	0.962	0.332
	5-HT	0.182	1	0.182	0.269	0.606
Ethanol * Stress * Housing	NE	38.130	1	38.130	4.804	0.033
	DOPAC	0.053	1	0.053	0.011	0.915
	5-HIAA	0.243	1	0.243	0.264	0.610
	DA	0.563	1	0.563	0.006	0.939
	HVA	2.025	1	2.025	1.109	0.298
	5-HT	0.697	1	0.697	1.029	0.316
Error	NE	365.078	46	7.936		
	DOPAC	212.078	46	4.610		
	5-HIAA	42.331	46	0.920		
	DA	4417.274	46	96.028		
	HVA	83.996	46	1.826		
	5-HT	31.152	46	0.677		

Table 80 – ANOVA Table for DA in the Ventral Tegmental Area

Source	Sum of Squares	df	Mean Square	F	Sig.
Ethanol	640.213	1	640.213	6.559	0.013
Stress	190.107	1	190.107	1.948	0.169
Housing	12.575	1	12.575	0.129	0.721
Ethanol * Stress	1.061	1	1.061	0.011	0.917
Ethanol * Housing	51.571	1	51.571	0.528	0.471
Stress * Housing	301.200	1	301.200	3.086	0.085
Ethanol * Stress * Housing	17.233	1	17.233	0.177	0.676
Error	5173.517	53	97.614		

Table 81 – ANOVA Table for HVA in the Ventral Tegmental Area

Source	Sum of Squares	df	Mean Square	F	Sig.
Ethanol	8.396	1	8.396	4.498	0.039
Stress	0.098	1	0.098	0.052	0.820
Housing	1.800	1	1.800	0.964	0.331
Ethanol * Stress	1.142	1	1.142	0.612	0.438
Ethanol * Housing	0.480	1	0.480	0.257	0.614
Stress * Housing	3.358	1	3.358	1.799	0.186
Ethanol * Stress * Housing	0.283	1	0.283	0.152	0.698
Error	97.064	52	1.867		

Table 82 – ANOVA Table for NE in the Ventral Tegmental Area

Source	Sum of Squares	df	Mean Square	F	Sig.
Ethanol	34.247	1	34.247	4.484	0.039
Stress	17.692	1	17.692	2.316	0.134
Housing	11.197	1	11.197	1.466	0.232
Ethanol * Stress	0.140	1	0.140	0.018	0.893
Ethanol * Housing	0.060	1	0.060	0.008	0.930
Stress * Housing	28.116	1	28.116	3.681	0.061
Ethanol * Stress * Housing	30.583	1	30.583	4.004	0.051
Error	389.520	51	7.638		

Table 83 – Correlation Matrix for Neurochemicals in the Prefrontal Cortex

Neurochemical		NE	DOPAC	5-HIAA	DA	HVA	5-HT
NE	Pearson Correlation	1	0.326	0.266	0.414	0.400	0.437
	Sig. (2-tailed)		0.011	0.034	0.001	0.001	0.000
DOPAC	Pearson Correlation	0.326	1	0.214	0.735	0.738	0.470
	Sig. (2-tailed)	0.011		0.086	0.000	0.000	0.000
5-HIAA	Pearson Correlation	0.266	0.214	1	0.335	0.372	0.388
	Sig. (2-tailed)	0.034	0.086		0.006	0.002	0.001
DA	Pearson Correlation	0.414	0.735	0.335	1	0.653	0.446
	Sig. (2-tailed)	0.001	0.000	0.006		0.000	0.000
HVA	Pearson Correlation	0.400	0.738	0.372	0.653	1	0.382
	Sig. (2-tailed)	0.001	0.000	0.002	0.000		0.002
5-HT	Pearson Correlation	0.437	0.470	0.388	0.446	0.382	1
	Sig. (2-tailed)	0.000	0.000	0.001	0.000	0.002	

Table 84a– MANOVA Table for Neurochemicals in the Prefrontal Cortex

Multivariate Test					
Effect	*V* Value	F	df	Error df	Sig.
Ethanol	0.304	3.063	6	42	0.014
Stress	0.145	1.186	6	42	0.333
Housing	0.184	1.579	6	42	0.177
Ethanol * Stress	0.034	0.243	6	42	0.959
Ethanol * Housing	0.209	1.850	6	42	0.113
Stress * Housing	0.072	0.542	6	42	0.773
Ethanol * Stress * Housing	0.107	0.842	6	42	0.545

Table 84b – MANOVA Table for Neurochemicals in the Prefrontal Cortex

Test of Between-Subjects Effects						
Source	**Dependent Variable**	**Sum of Squares**	**df**	**Mean Square**	**F**	**Sig.**
Ethanol	NE	1.600	1	1.600	1.223	0.274
	DOPAC	0.119	1	0.119	0.632	0.431
	5-HIAA	0.551	1	0.551	2.329	0.134
	DA	0.046	1	0.046	0.052	0.820
	HVA	0.913	1	0.913	3.090	0.085
	5-HT	0.742	1	0.742	3.030	0.088
Stress	NE	0.892	1	0.892	0.682	0.413
	DOPAC	0.012	1	0.012	0.061	0.805
	5-HIAA	0.066	1	0.066	0.281	0.599
	DA	0.006	1	0.006	0.007	0.935
	HVA	0.001	1	0.001	0.002	0.964
	5-HT	0.792	1	0.792	3.235	0.079
Housing	NE	3.240	1	3.240	2.477	0.122
	DOPAC	0.081	1	0.081	0.430	0.515
	5-HIAA	0.020	1	0.020	0.084	0.773
	DA	2.163	1	2.163	2.462	0.123
	HVA	0.001	1	0.001	0.005	0.946
	5-HT	1.262	1	1.262	5.154	0.028
Ethanol * Stress	NE	0.472	1	0.472	0.361	0.551
	DOPAC	0.021	1	0.021	0.110	0.742
	5-HIAA	0.037	1	0.037	0.157	0.693
	DA	0.039	1	0.039	0.044	0.835
	HVA	0.091	1	0.091	0.309	0.581
	5-HT	0.049	1	0.049	0.199	0.657
Ethanol * Housing	NE	1.554	1	1.554	1.189	0.281
	DOPAC	0.781	1	0.781	4.149	0.047
	5-HIAA	0.462	1	0.462	1.951	0.169
	DA	2.007	1	2.007	2.285	0.137
	HVA	2.148	1	2.148	7.270	0.010
	5-HT	1.969	1	1.969	8.044	0.007
Stress * Housing	NE	1.272	1	1.272	0.973	0.329
	DOPAC	0.017	1	0.017	0.092	0.763
	5-HIAA	0.563	1	0.563	2.381	0.130
	DA	0.062	1	0.062	0.071	0.791
	HVA	0.028	1	0.028	0.096	0.758
	5-HT	0.224	1	0.224	0.913	0.344
Ethanol * Stress * Housing	NE	0.855	1	0.855	0.654	0.423
	DOPAC	0.102	1	0.102	0.541	0.466
	5-HIAA	0.119	1	0.119	0.501	0.483
	DA	2.730	1	2.730	3.108	0.084
	HVA	0.671	1	0.671	2.272	0.138
	5-HT	0.490	1	0.490	2.001	0.164
Error	NE	61.463	47	1.308		
	DOPAC	8.849	47	0.188		
	5-HIAA	11.120	47	0.237		
	DA	41.283	47	0.878		
	HVA	13.886	47	0.295		
	5-HT	11.505	47	0.245		

Table 85 – ANOVA Table for DOPAC in the Prefrontal Cortex

Source	Sum of Squares	df	Mean Square	F	Sig.
Ethanol	0.096	1	0.096	0.369	0.546
Stress	0.054	1	0.054	0.208	0.650
Housing	0.311	1	0.311	1.200	0.278
Ethanol * Stress	0.083	1	0.083	0.318	0.575
Ethanol * Housing	0.494	1	0.494	1.904	0.173
Stress * Housing	0.046	1	0.046	0.179	0.674
Ethanol * Stress * Housing	0.001	1	0.001	0.003	0.960
Error	14.777	57	0.259		

Table 86 – ANOVA Table for HVA in the Prefrontal Cortex

Source	Sum of Squares	df	Mean Square	F	Sig.
Ethanol	0.766	1	0.766	2.169	0.146
Stress	0.026	1	0.026	0.074	0.787
Housing	0.494	1	0.494	1.398	0.242
Ethanol * Stress	0.001	1	0.001	0.004	0.953
Ethanol * Housing	2.583	1	2.583	7.315	0.009
Stress * Housing	0.228	1	0.228	0.646	0.425
Ethanol * Stress * Housing	0.170	1	0.170	0.482	0.490
Error	20.832	59	0.353		

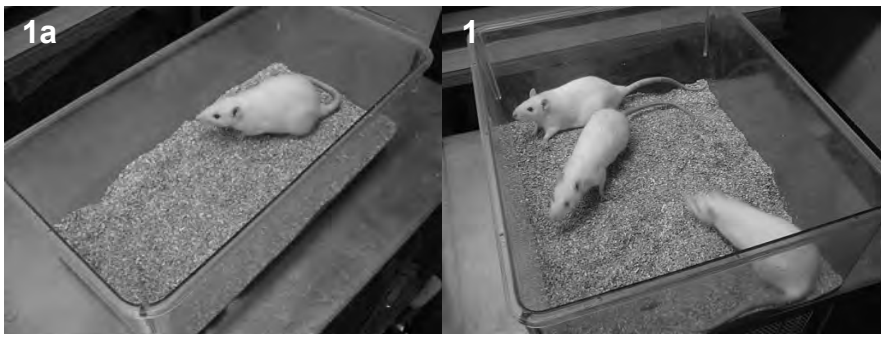
Table 87 – ANOVA Table for 5-HT in the Prefrontal Cortex

Source	Sum of Squares	df	Mean Square	F	Sig.
Ethanol	1.838	1	1.838	2.944	0.091
Stress	0.157	1	0.157	0.251	0.618
Housing	2.965	1	2.965	4.751	0.033
Ethanol * Stress	0.055	1	0.055	0.088	0.768
Ethanol * Housing	4.933	1	4.933	7.903	0.007
Stress * Housing	0.144	1	0.144	0.231	0.633
Ethanol * Stress * Housing	0.829	1	0.829	1.328	0.254
Error	36.826	59	0.624		

Appendix B:

Figure Appendix

Figure 1 – Isolated and Socially Enriched Housing Conditions*



*Cage lids removed for clarity

Figure 2 – Two-Bottle Choice Setup for Ethanol Self-Administration

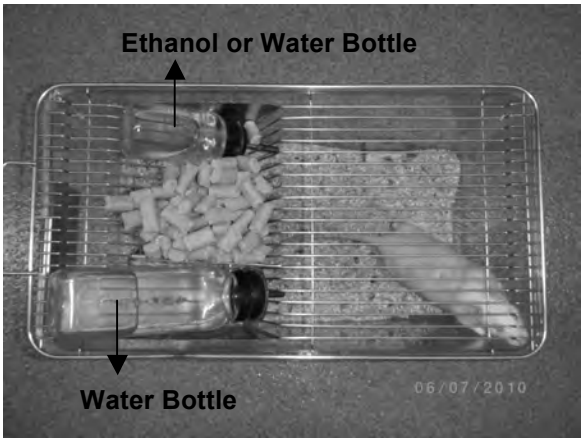


Figure 3 – Operant Liquid Self-Administration Setup for Ethanol Self-Administration

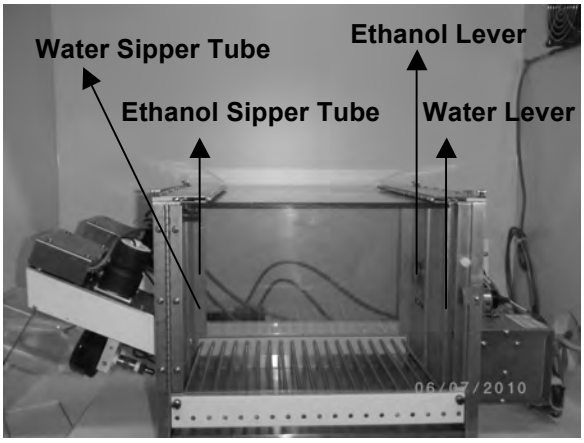
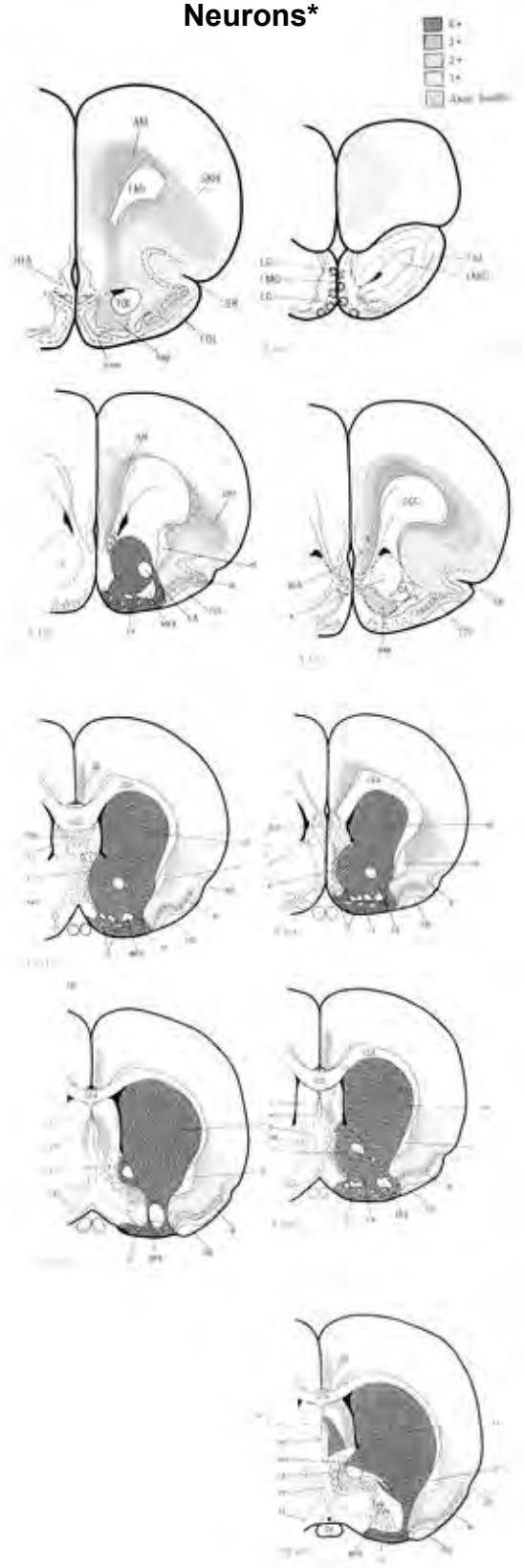


Figure 4 – Microdissected Regions Based on Density of DA-Containing Neurons*



*Darker shaded areas represent greater density of DA-containing neurons.

Figure 5 – Open Field Locomotor Setup

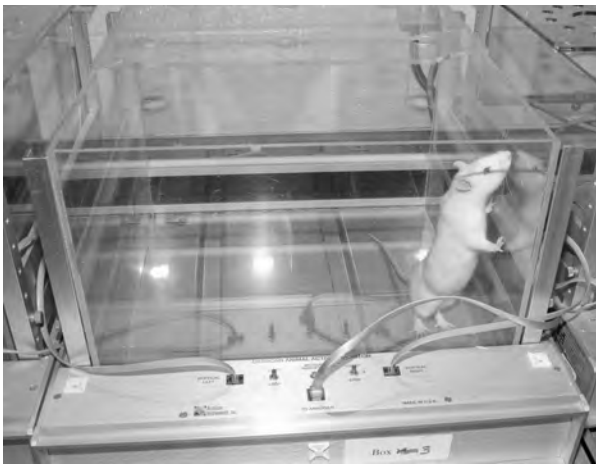


Figure 6 – Rotarod Setup

Figure 7 – Forced Swim Test Setup

Figure 8 – Serum Corticosterone Concentration

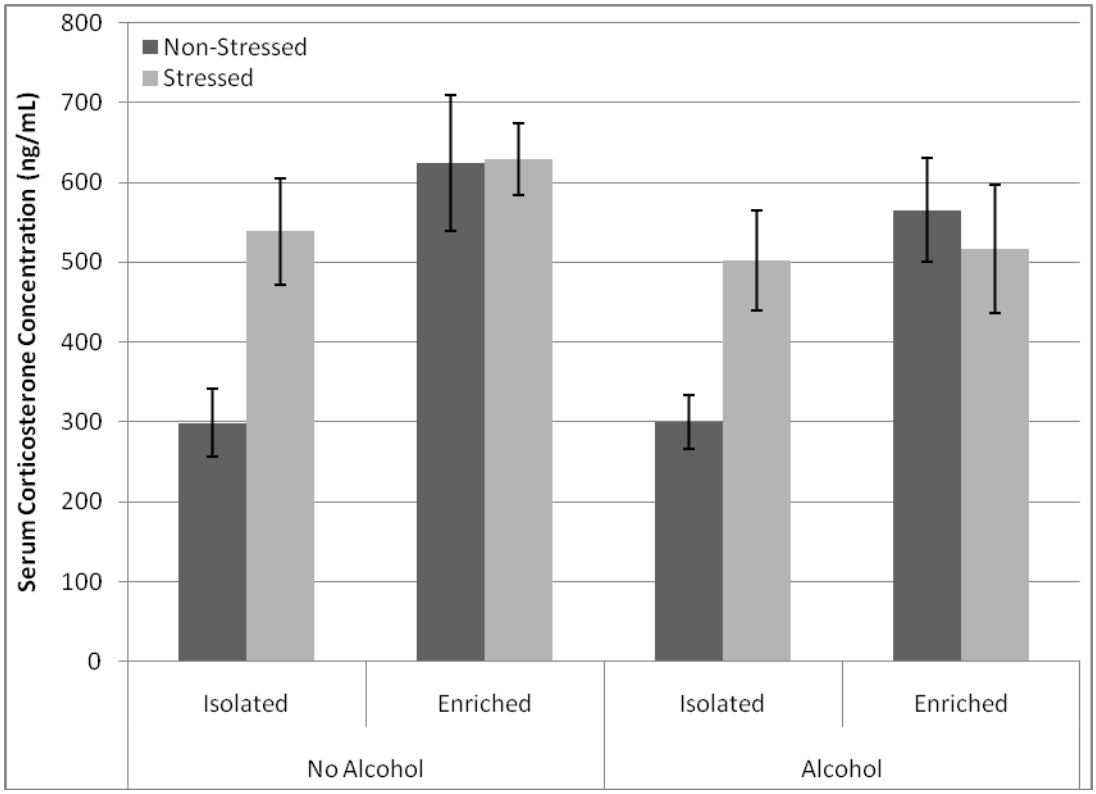


Figure 9 – Changes in Body Weight over Time

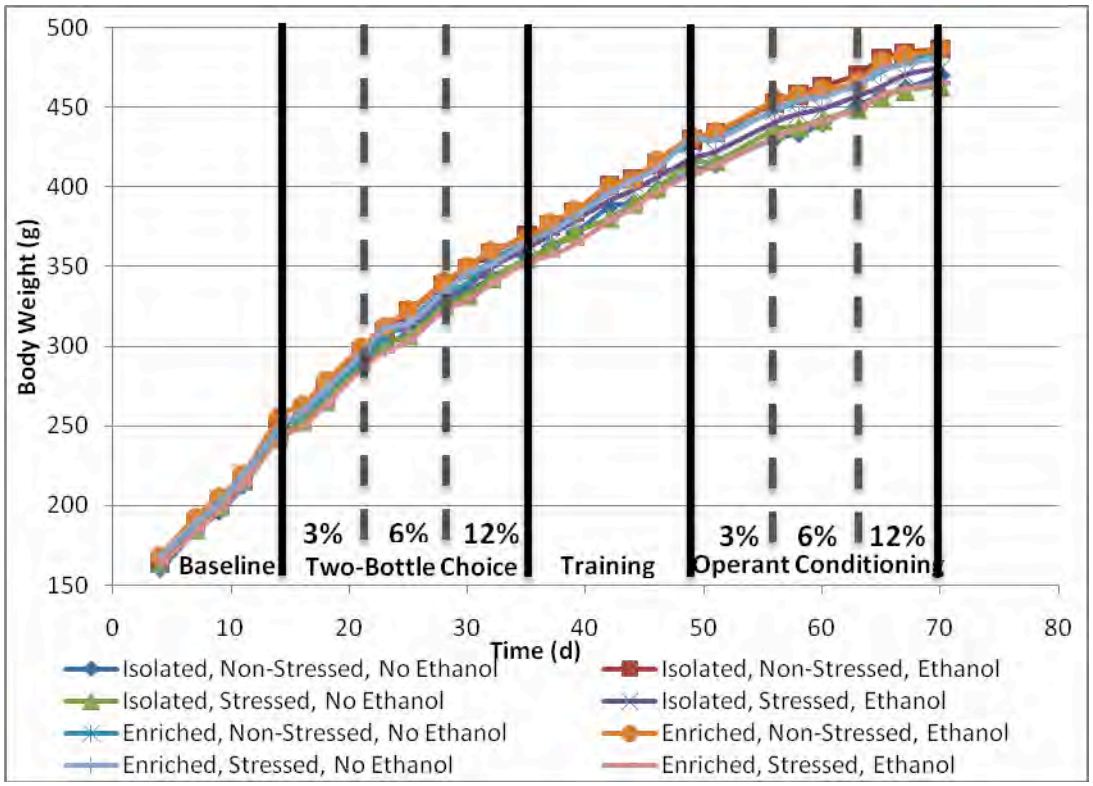


Figure 10 – Body Weight during Baseline Phase

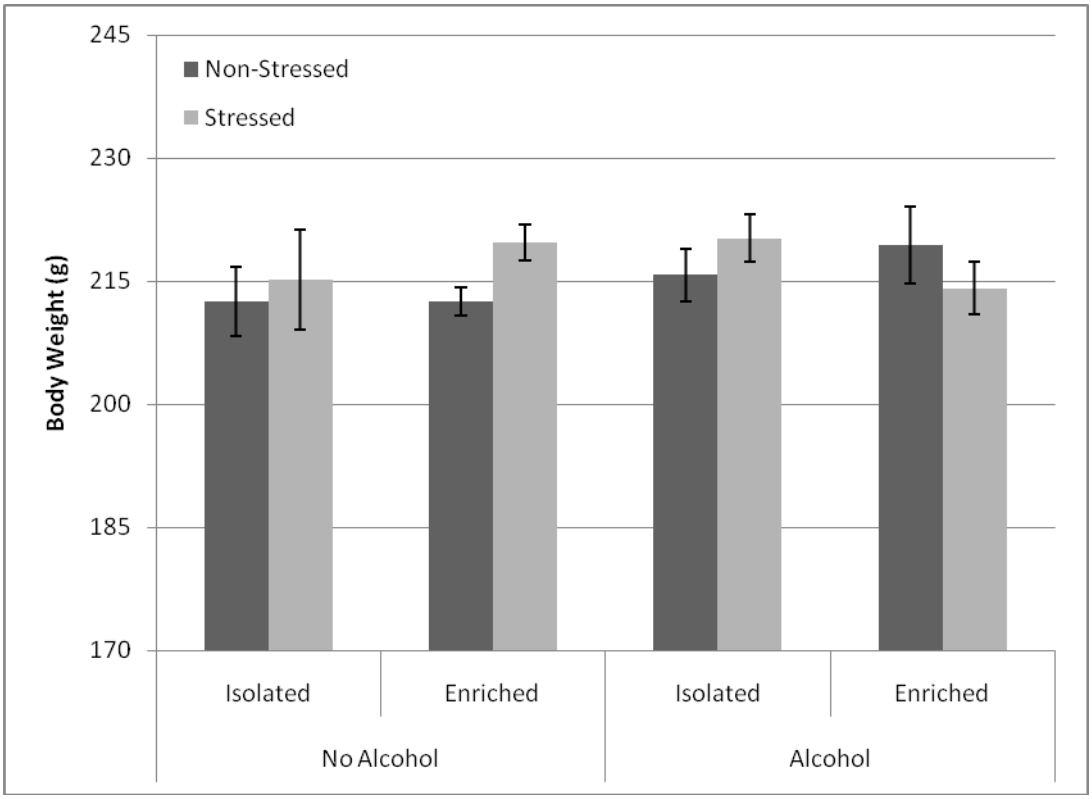


Figure 11 – Body Weight during Two-Bottle Choice Phase

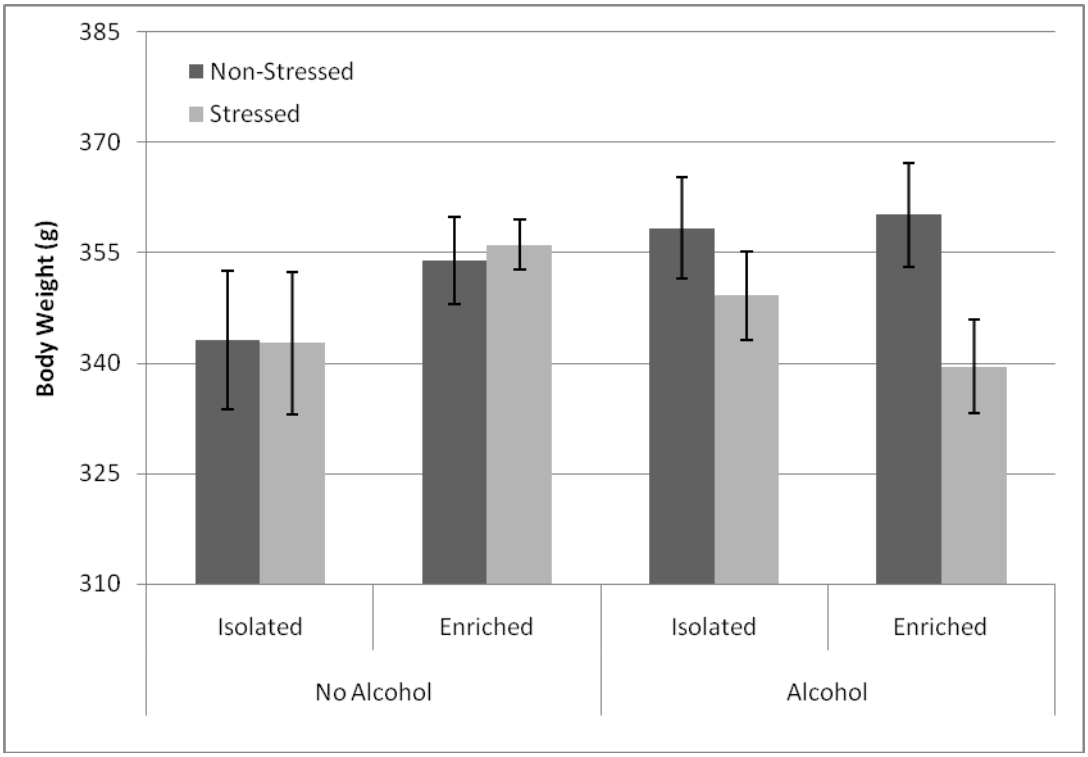


Figure 12 – Body Weight during Operant Liquid Self-Administration Phase

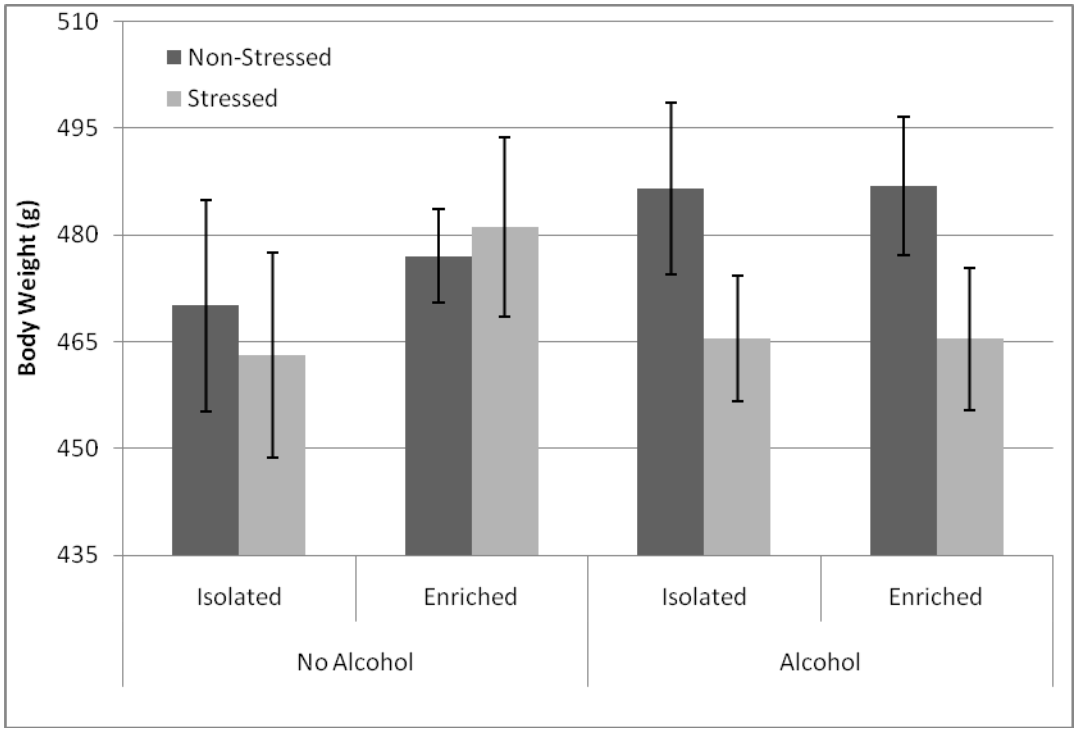


Figure 13 – Serum Ethanol Concentration

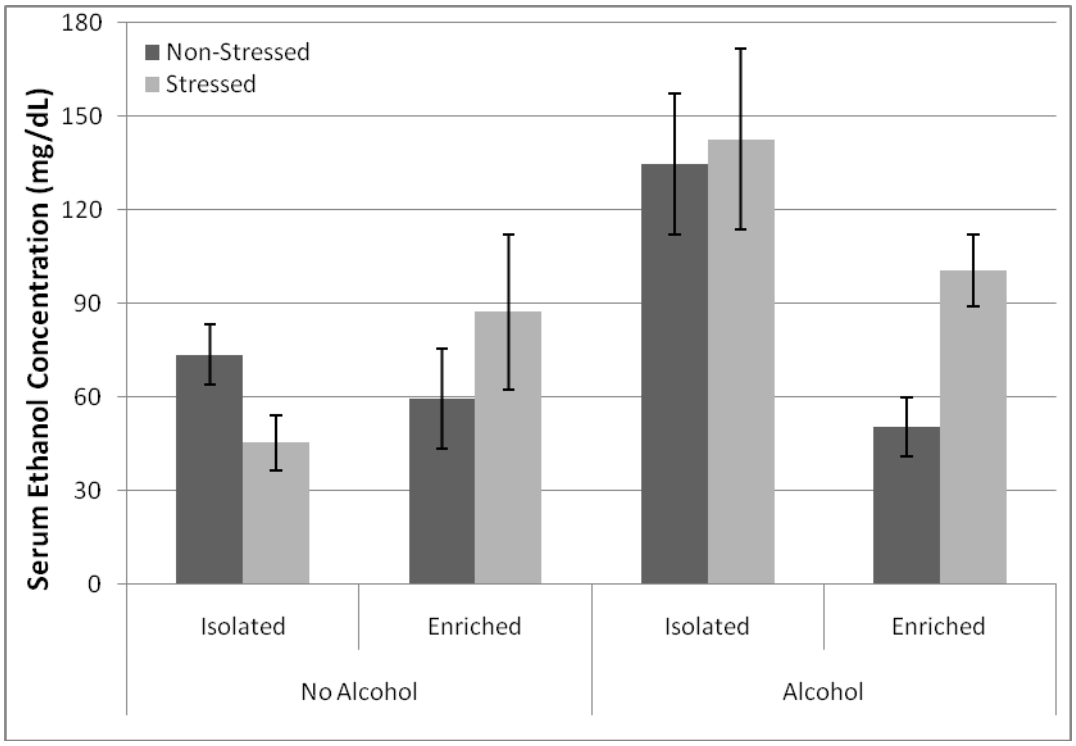


Figure 14 – Rotarod Performance during Baseline Phase

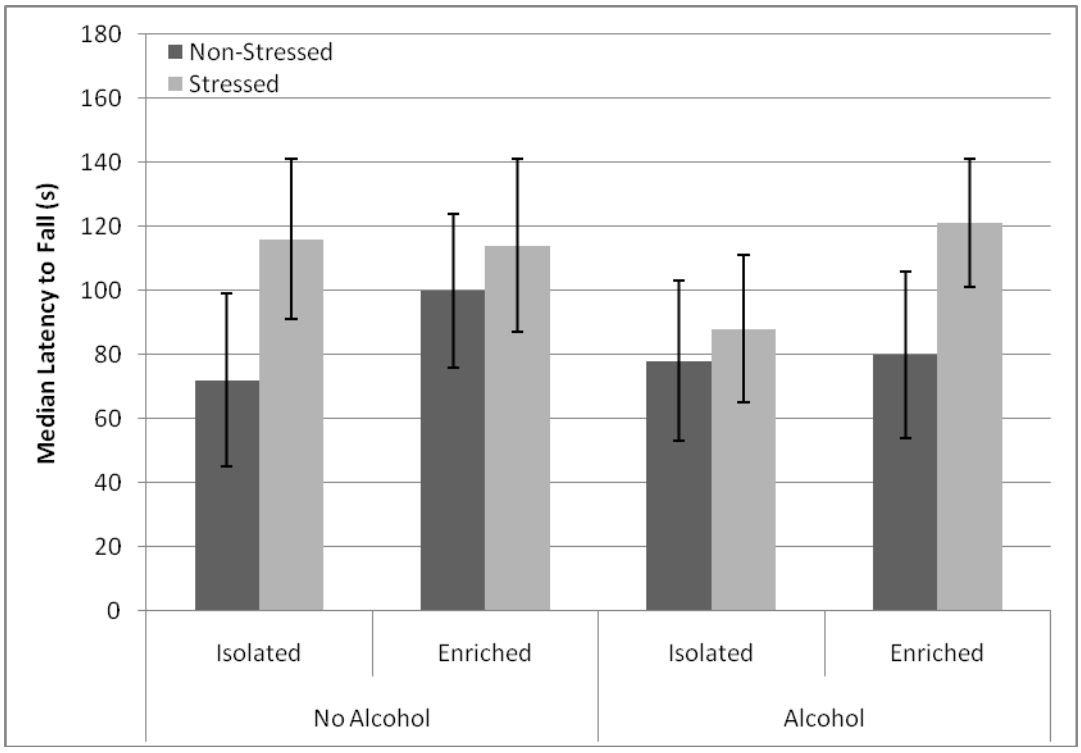


Figure 15 – Rotarod Performance during 2BC Phase

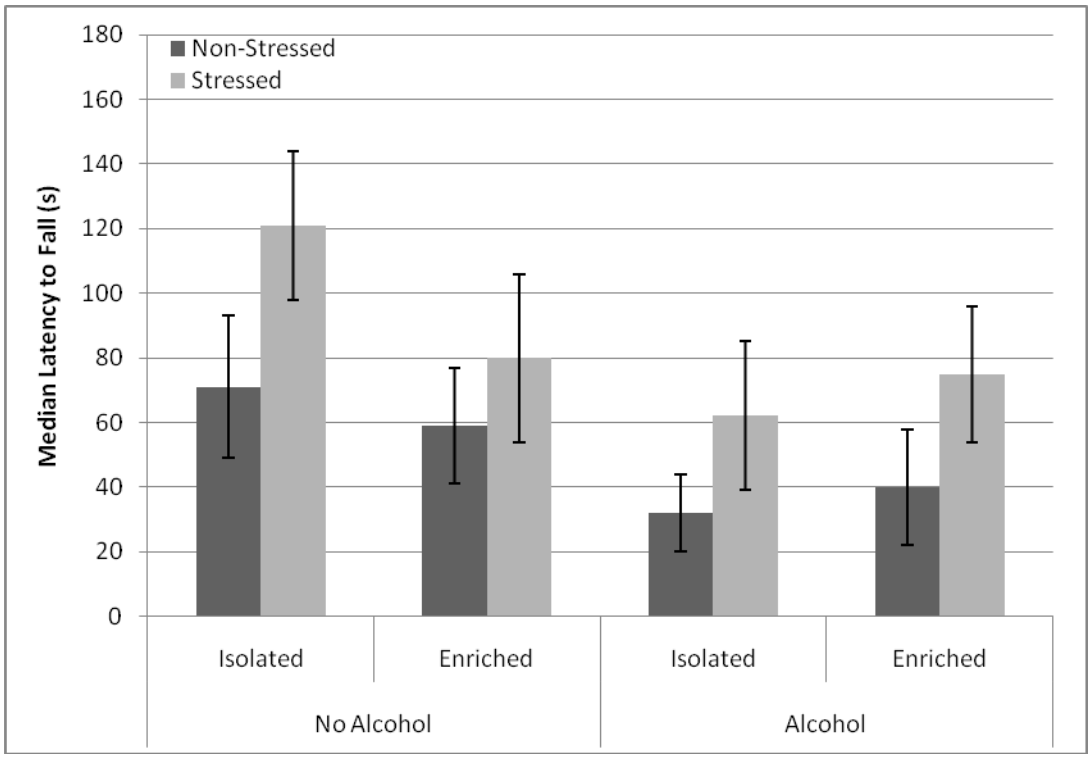


Figure 16 – Rotarod Performance during LSA Phase

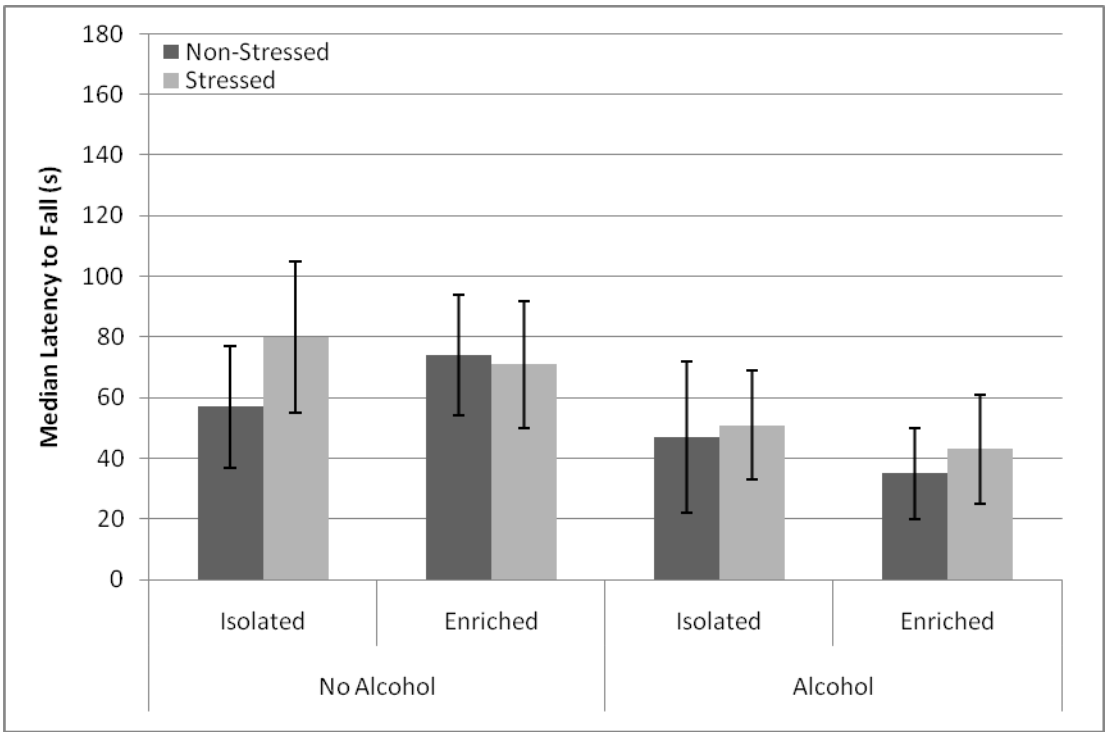


Figure 17 – Ethanol or Water Consumption over Time (2BC Phase)

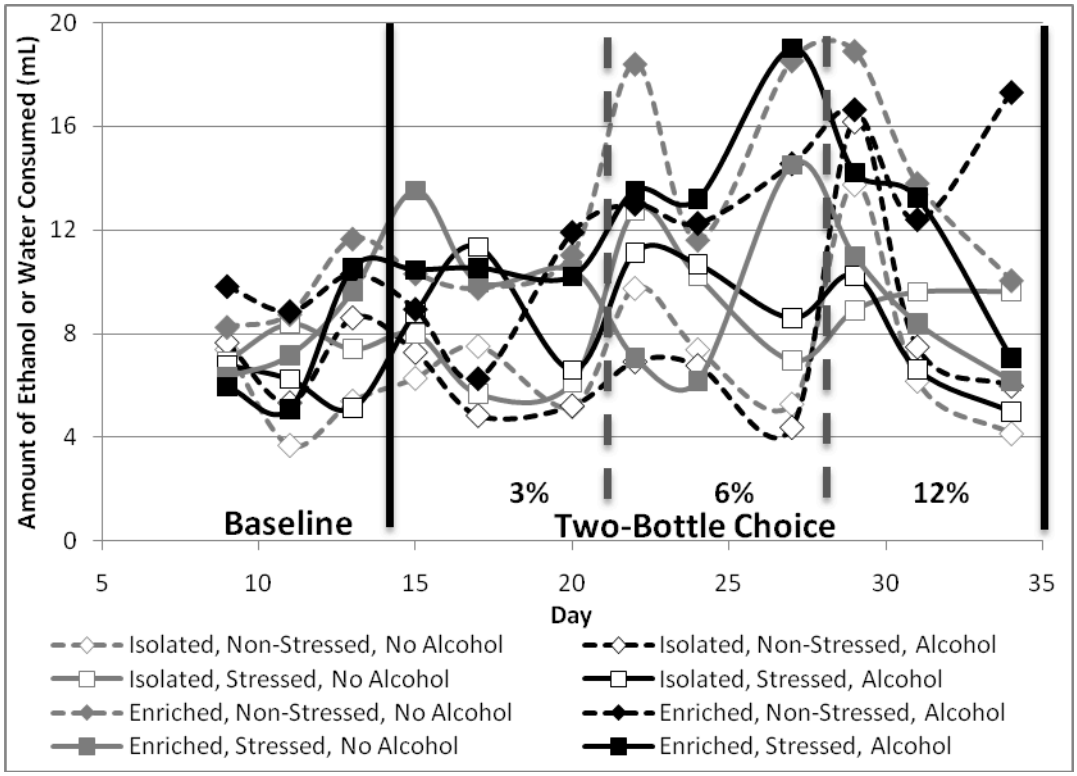


Figure 18 – "0% Ethanol" or Water Consumption (2BC Phase)

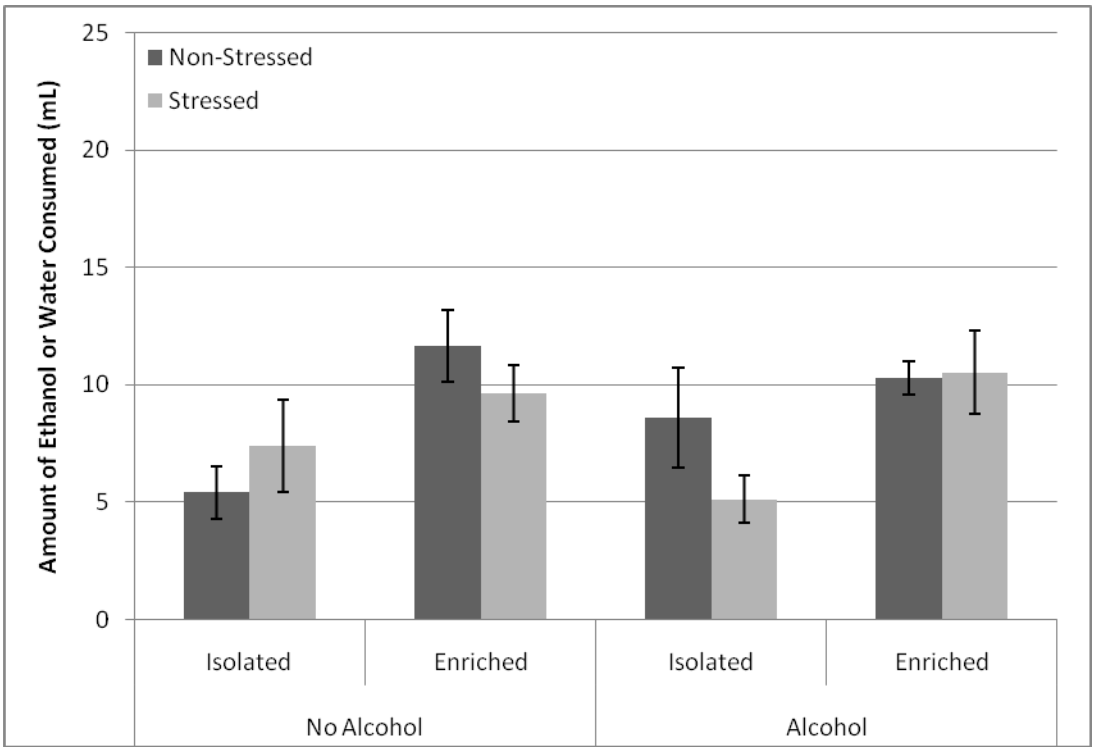


Figure 19 – 3% Ethanol or Water Consumption (2BC Phase)

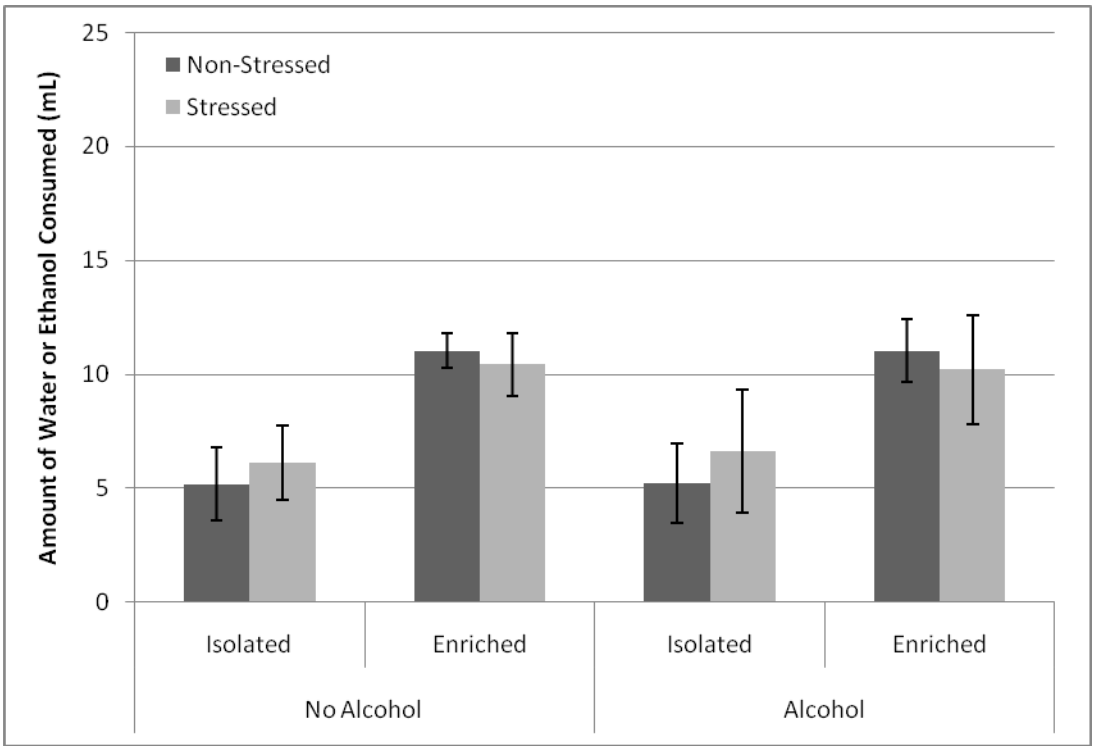


Figure 20 – 6% Ethanol or Water Consumption (2BC Phase)

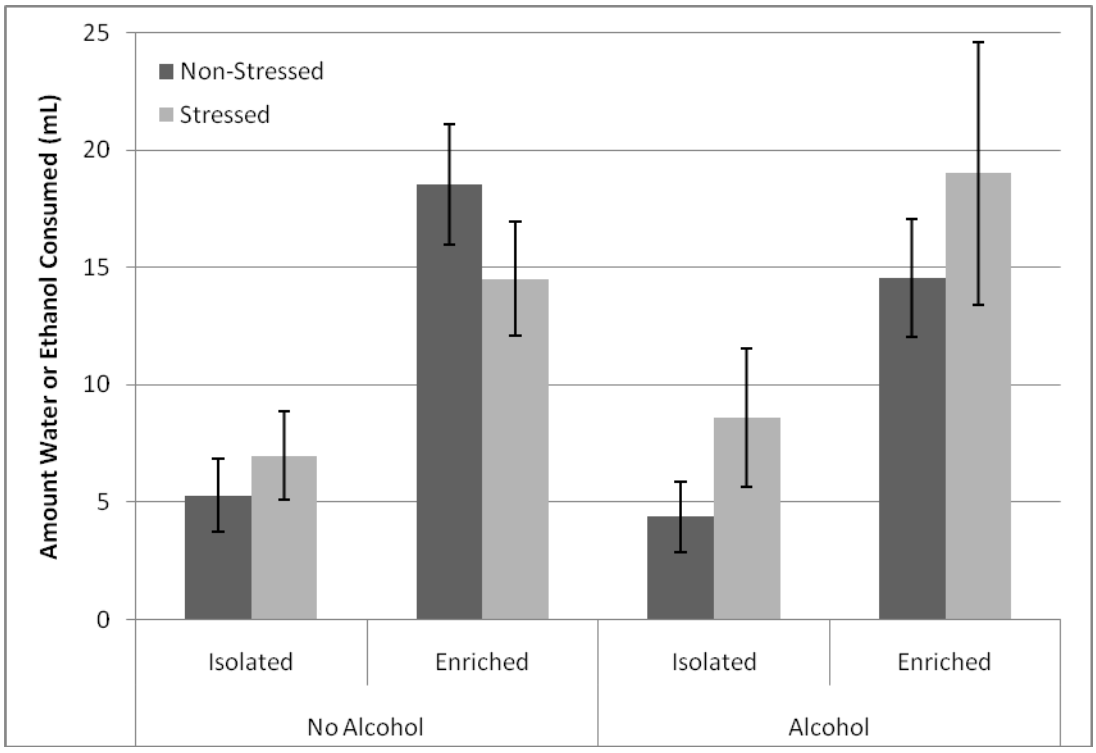


Figure 21 - 12% Ethanol or Water Consumption (2BC Phase)

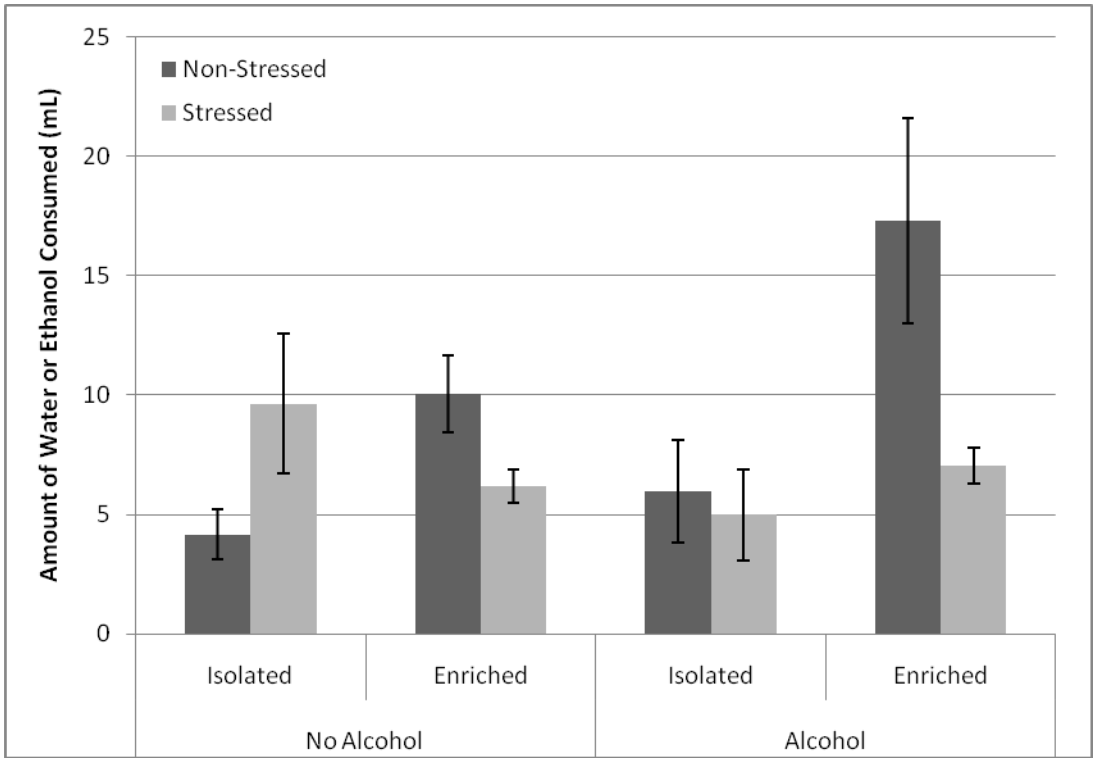


Figure 22 – Water Consumption over Time

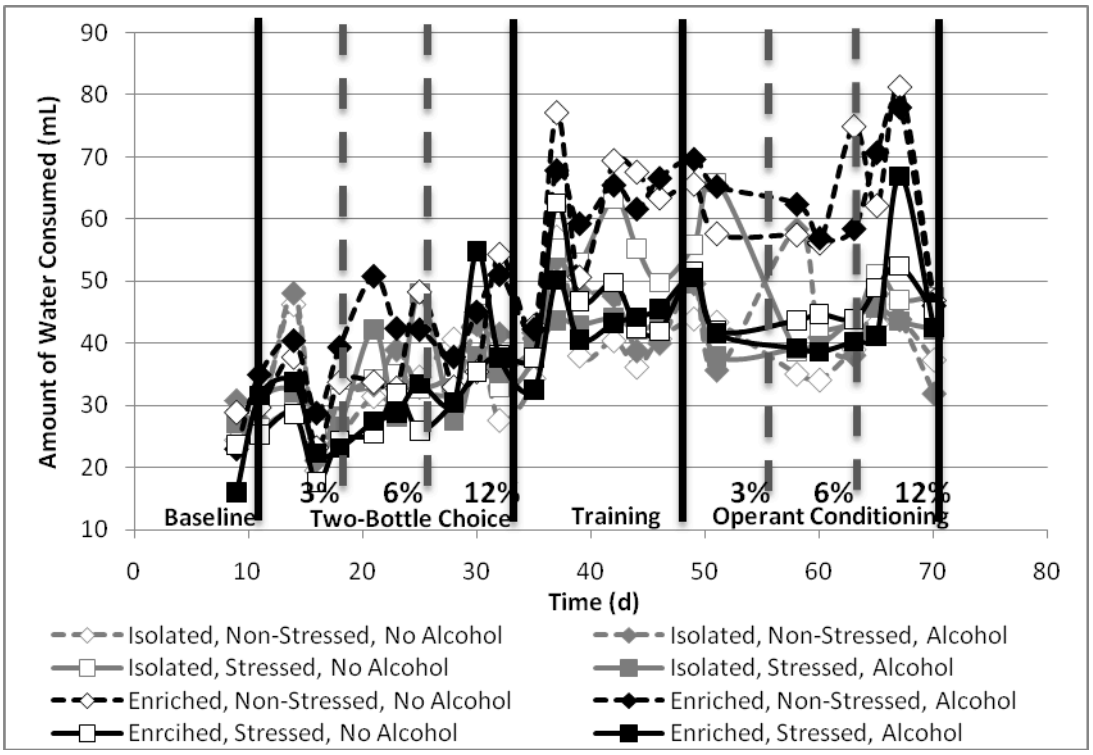


Figure 23 – Water Consumption, Baseline Phase

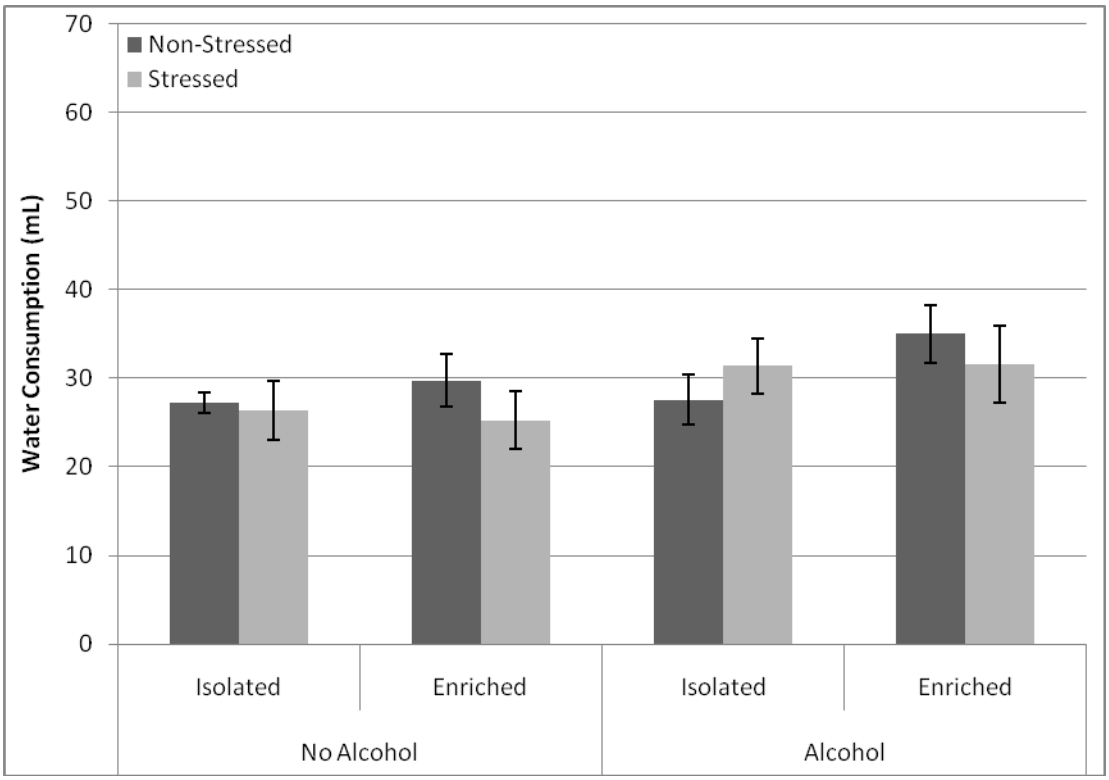


Figure 24 – Water Consumption, Two-Bottle Choice Phase

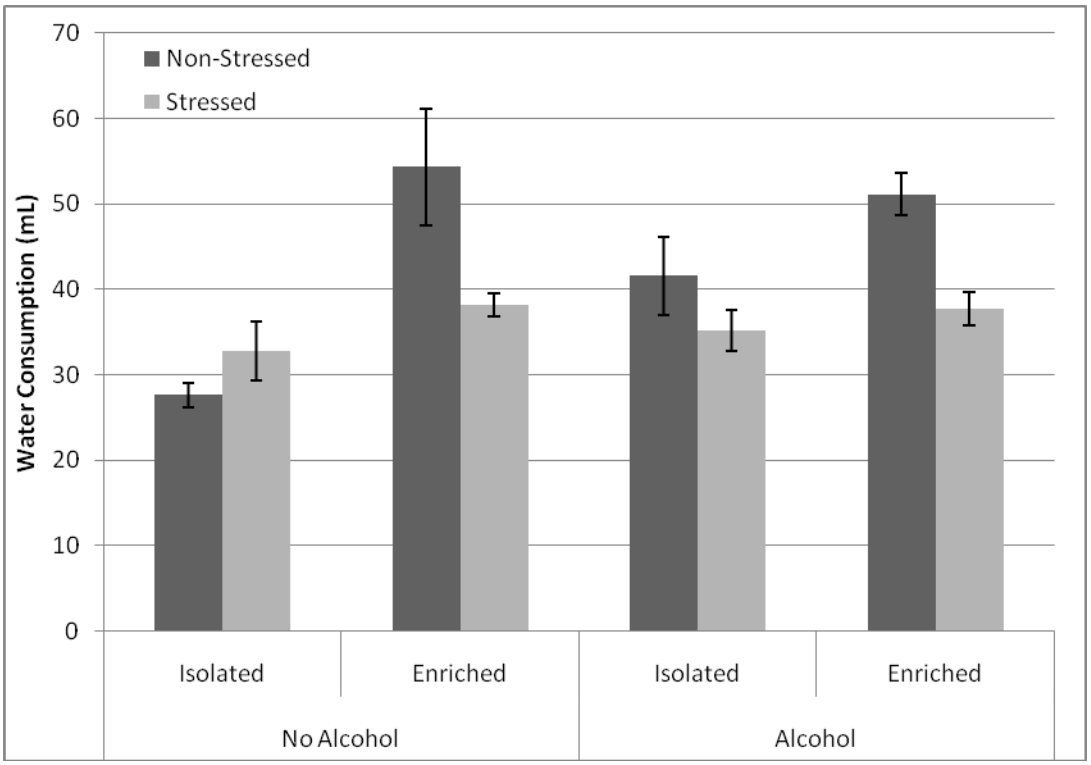


Figure 25 – Water Consumption, Liquid Self-Administration Phase

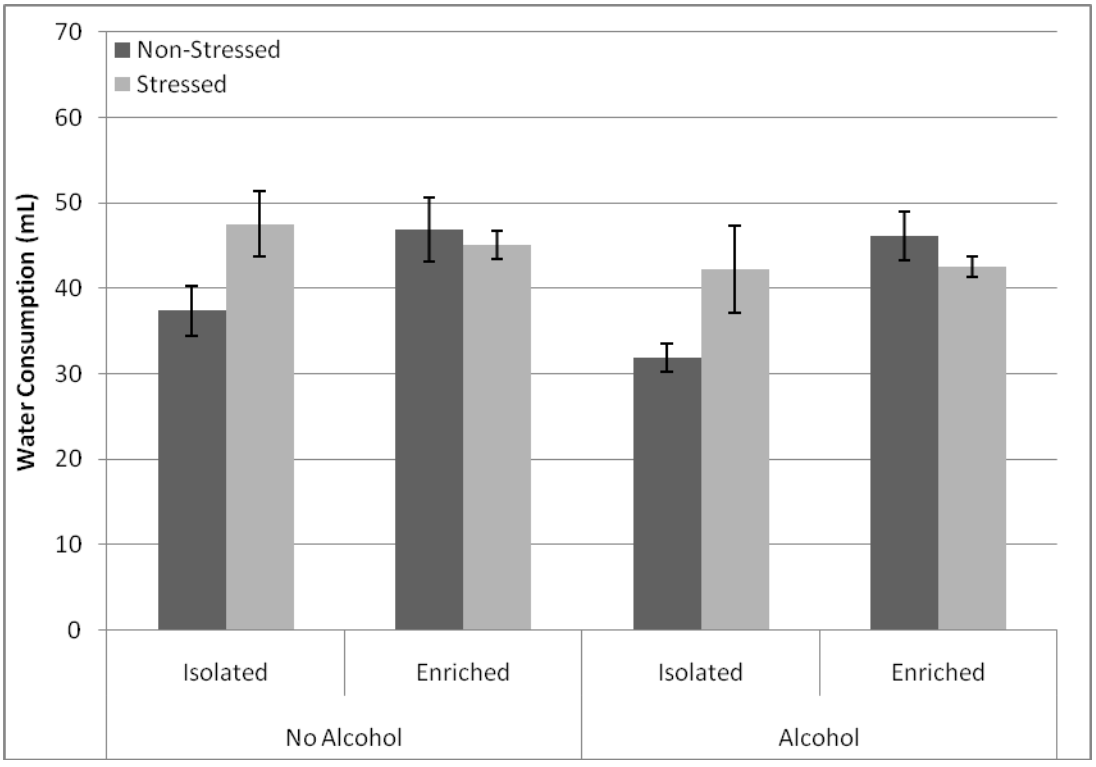


Figure 26 – Food Consumption over Time

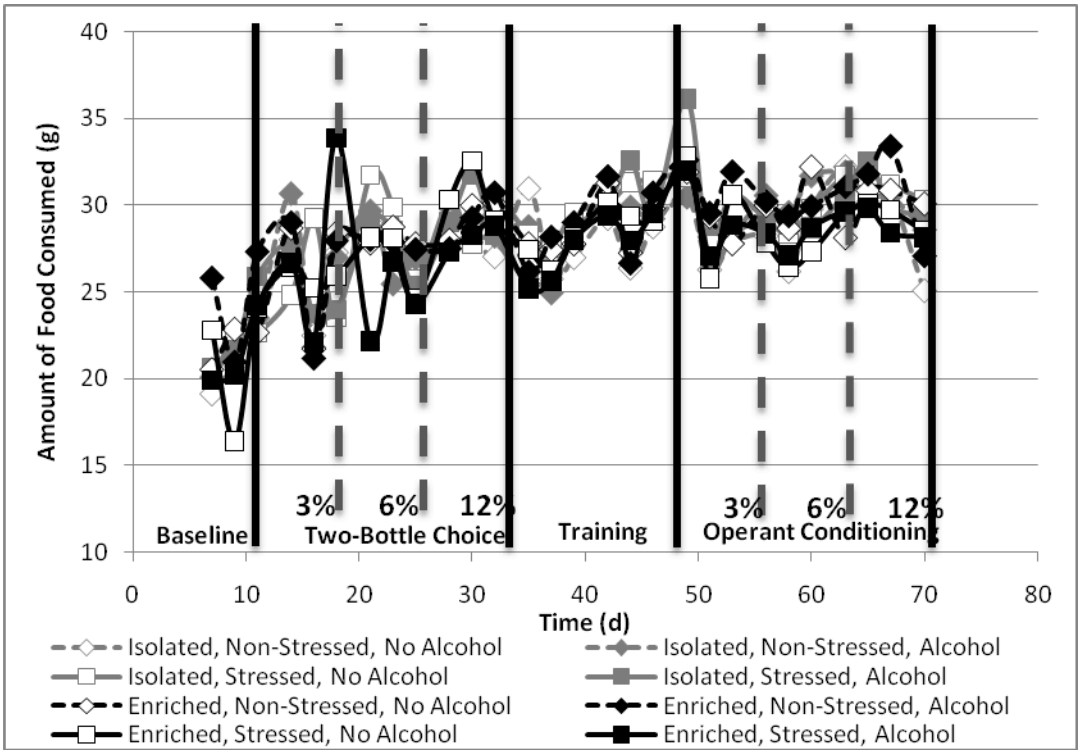


Figure 27 – Food Consumption, Baseline Phase

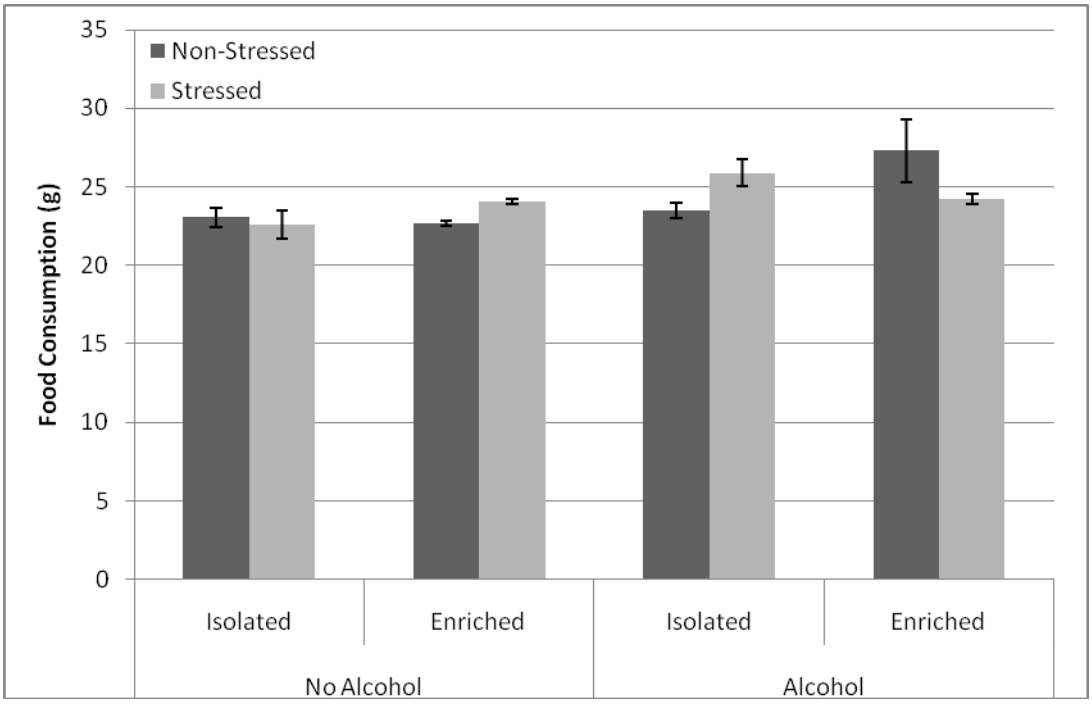


Figure 28 – Food Consumption, Two-Bottle Choice Phase

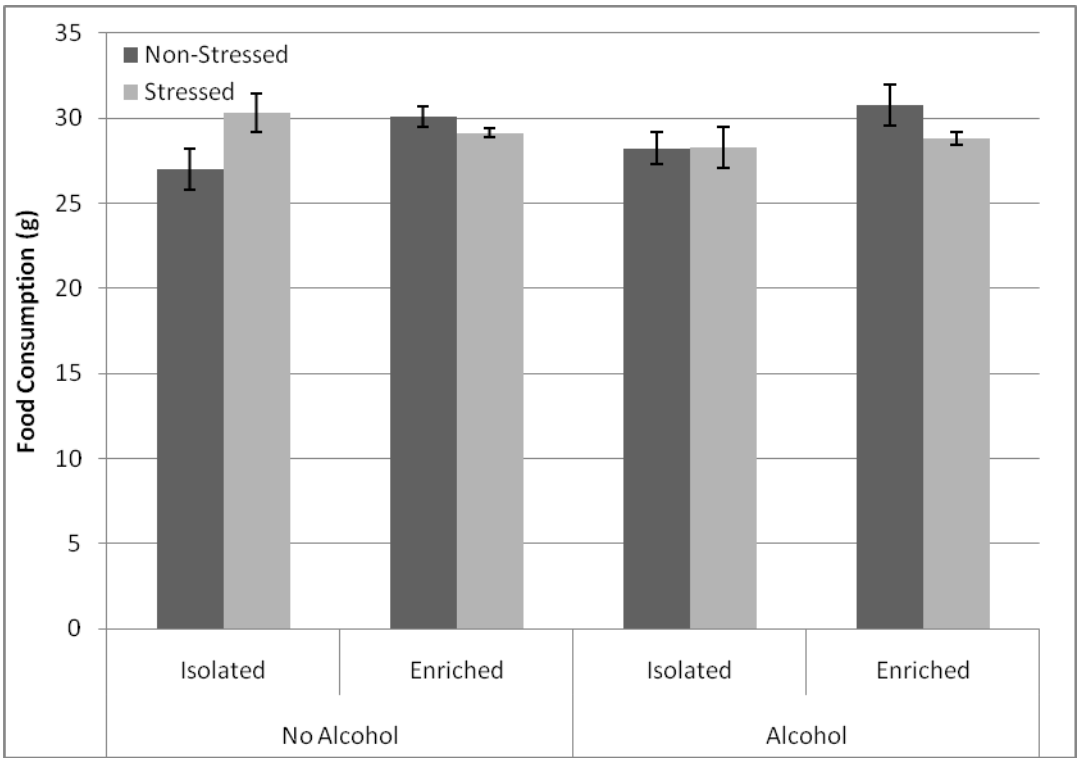


Figure 29 – Food Consumption, Liquid Self-Administration Phase

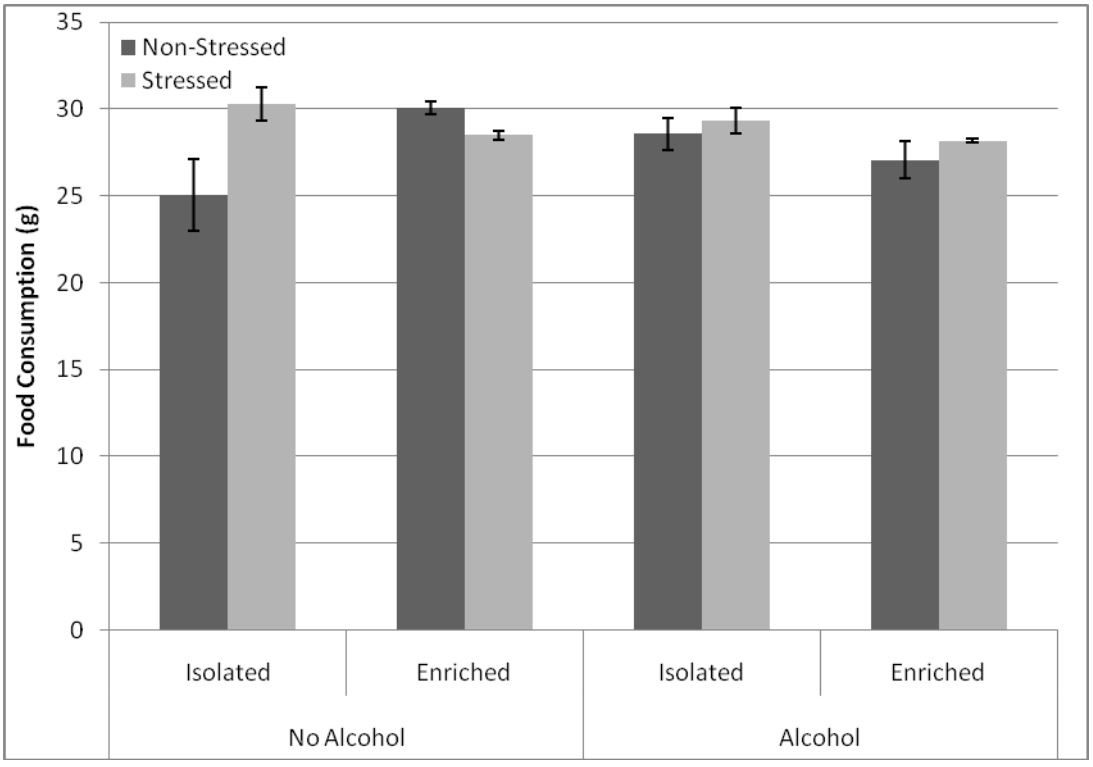


Figure 30 – Number of Licks for 3% Ethanol (LSA Phase)

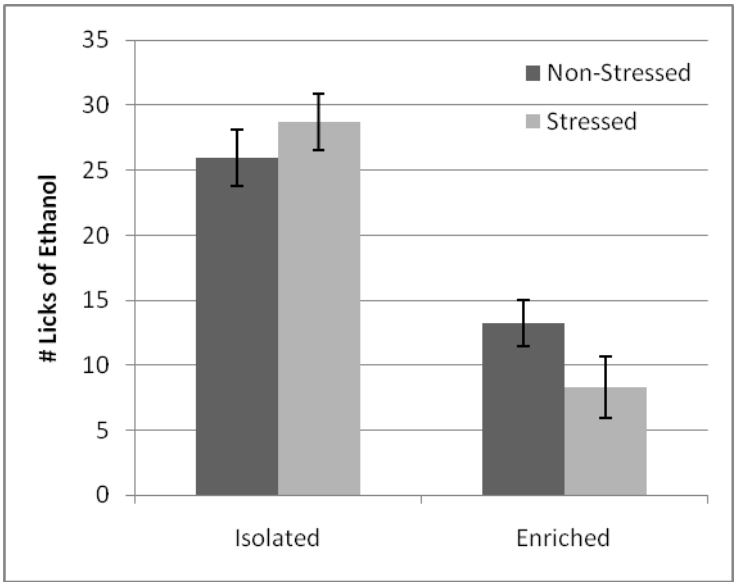


Figure 31 – Number of Lever Presses for 3% Ethanol (LSA Phase)

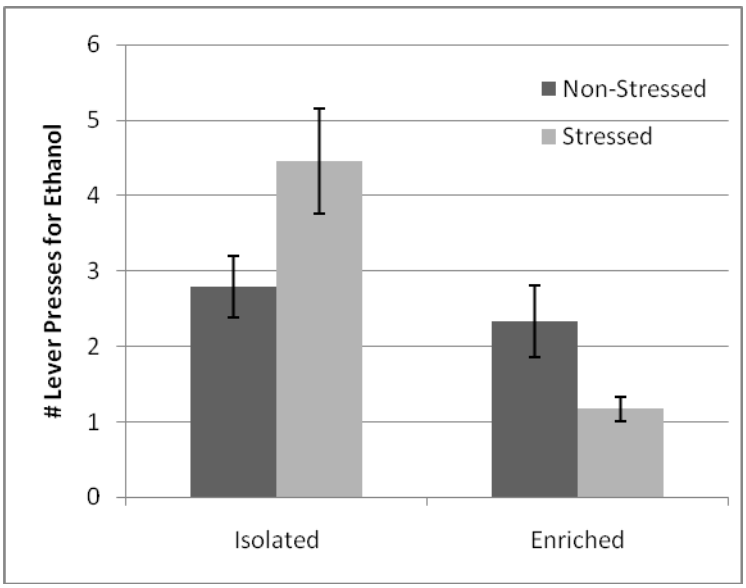


Figure 32 – Number of Lever Presses for Water (3% LSA Phase)

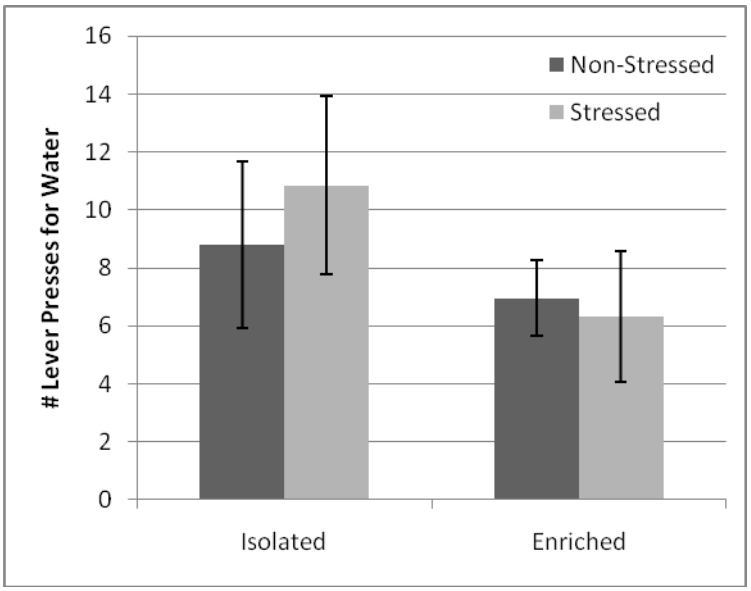


Figure 33 – Number of Licks for 6% Ethanol (LSA Phase)

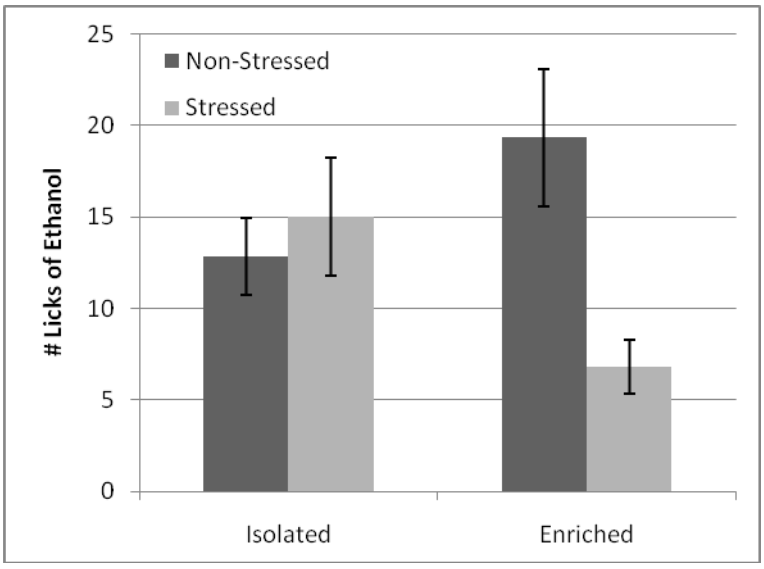


Figure 34 – Number of Lever Presses for 6% Ethanol (LSA Phase)

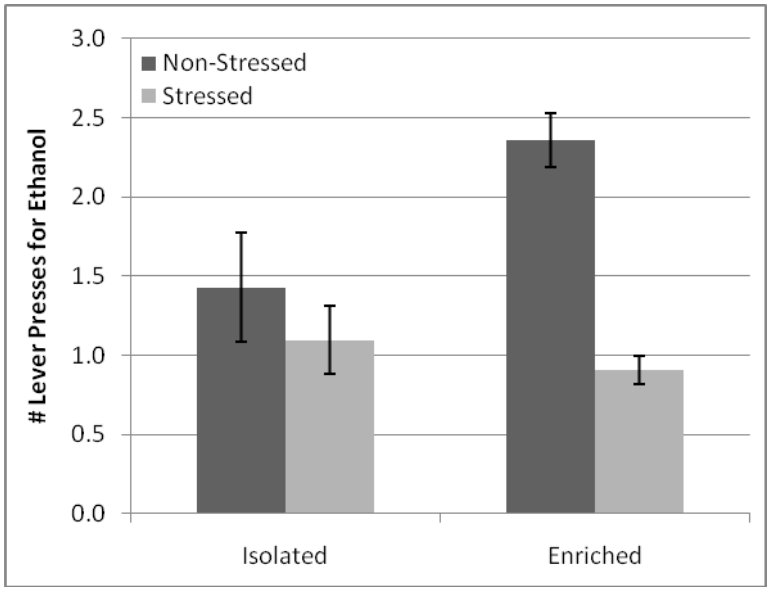


Figure 35 – Number of Lever Presses for Water (6% LSA Phase)

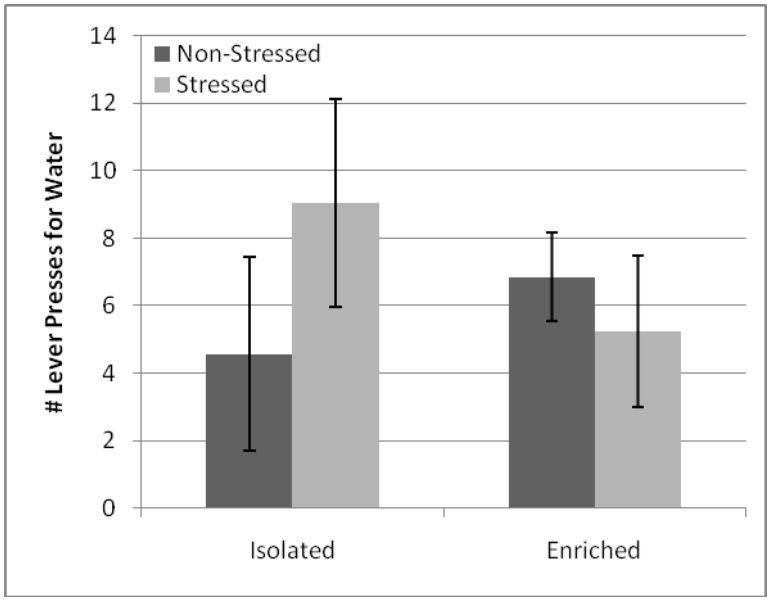


Figure 36 – Number of Licks for 12% Ethanol (LSA Phase)

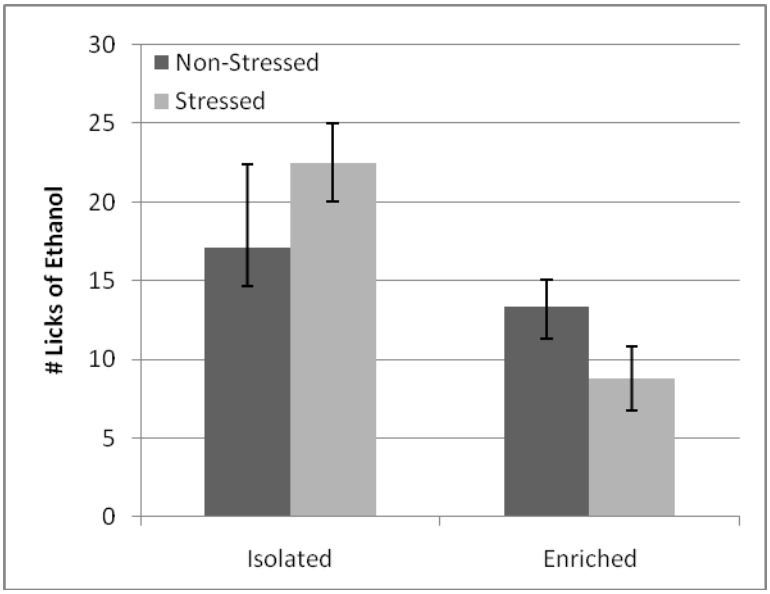


Figure 37 – Number of Lever Presses for 12% Ethanol (LSA Phase)

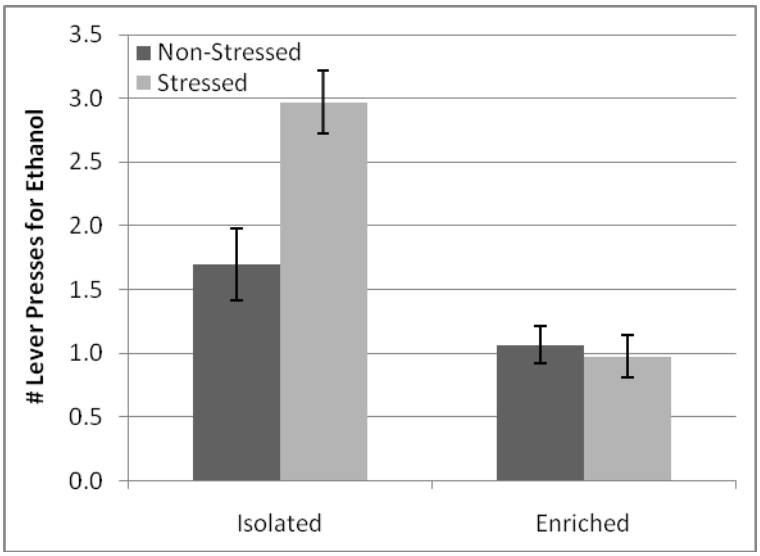


Figure 38 – Number of Lever Presses for Water (12% LSA Phase)

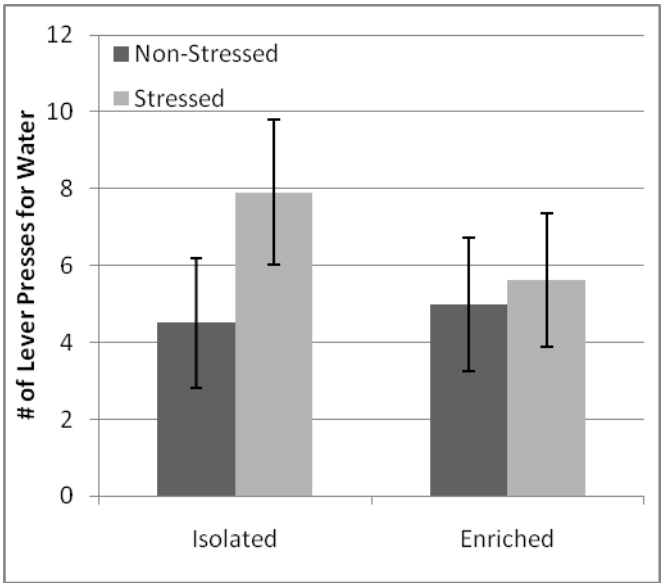


Figure 39 – Open Field Horizontal Activity, Baseline Phase

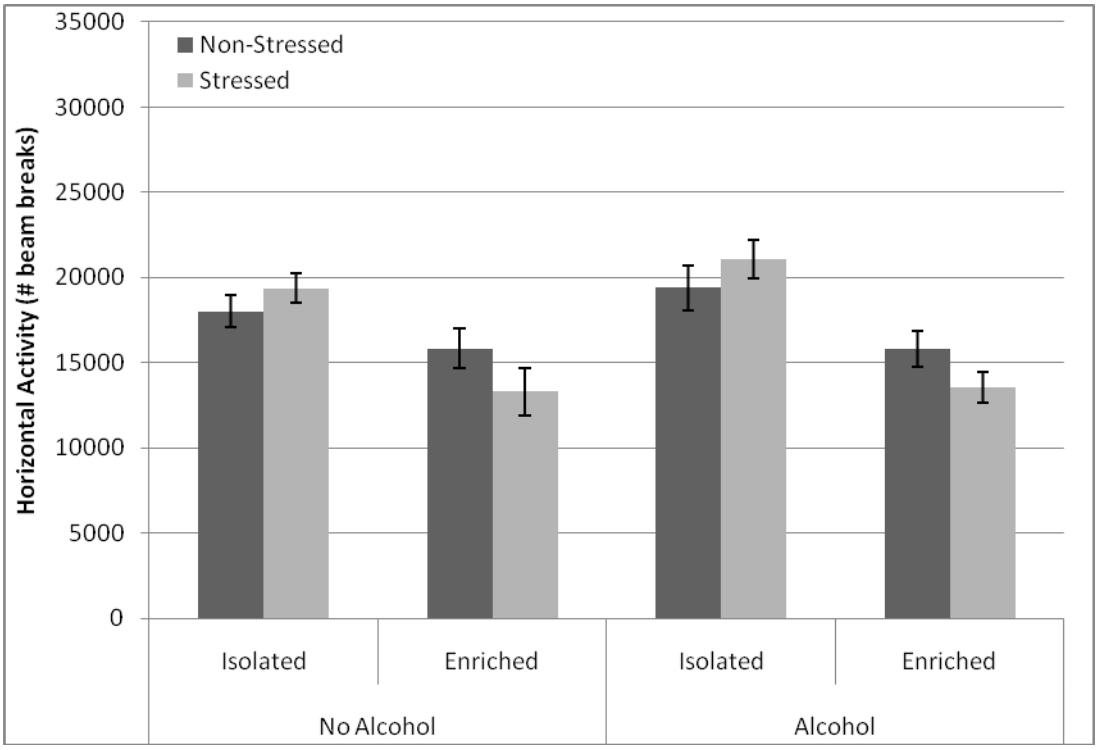


Figure 40 – Open Field Horizontal Activity, Two-Bottle Choice Phase

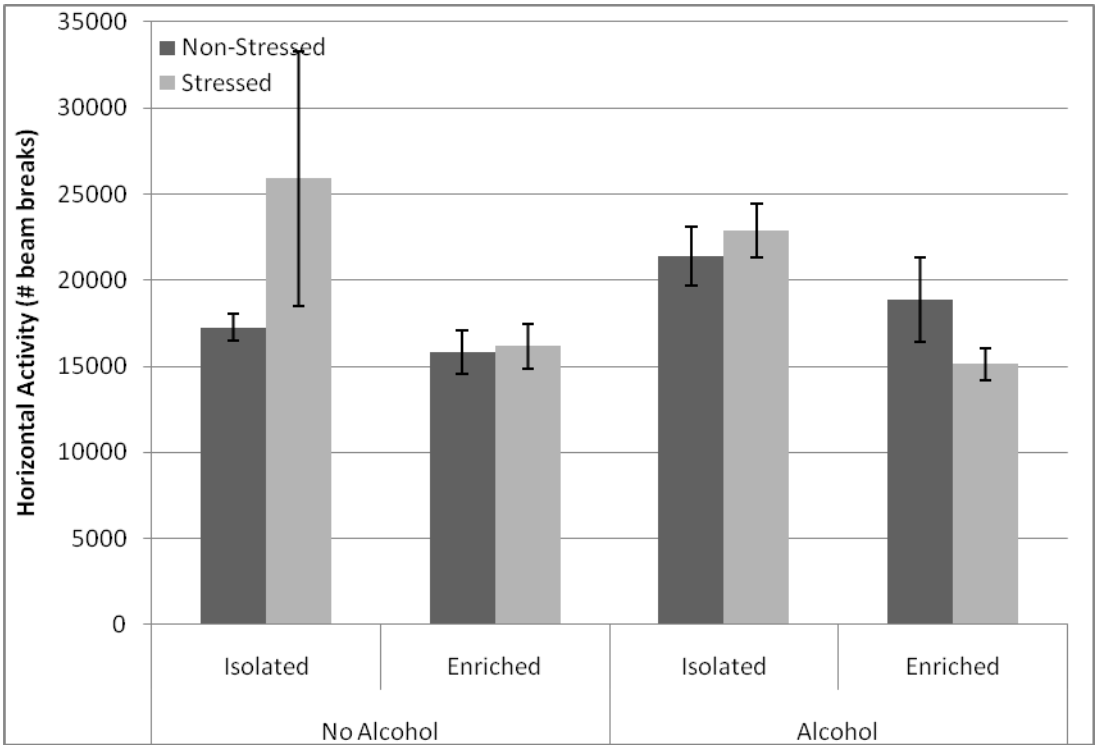


Figure 41 – Open Field Horizontal Activity, Liquid Self-Administration Phase

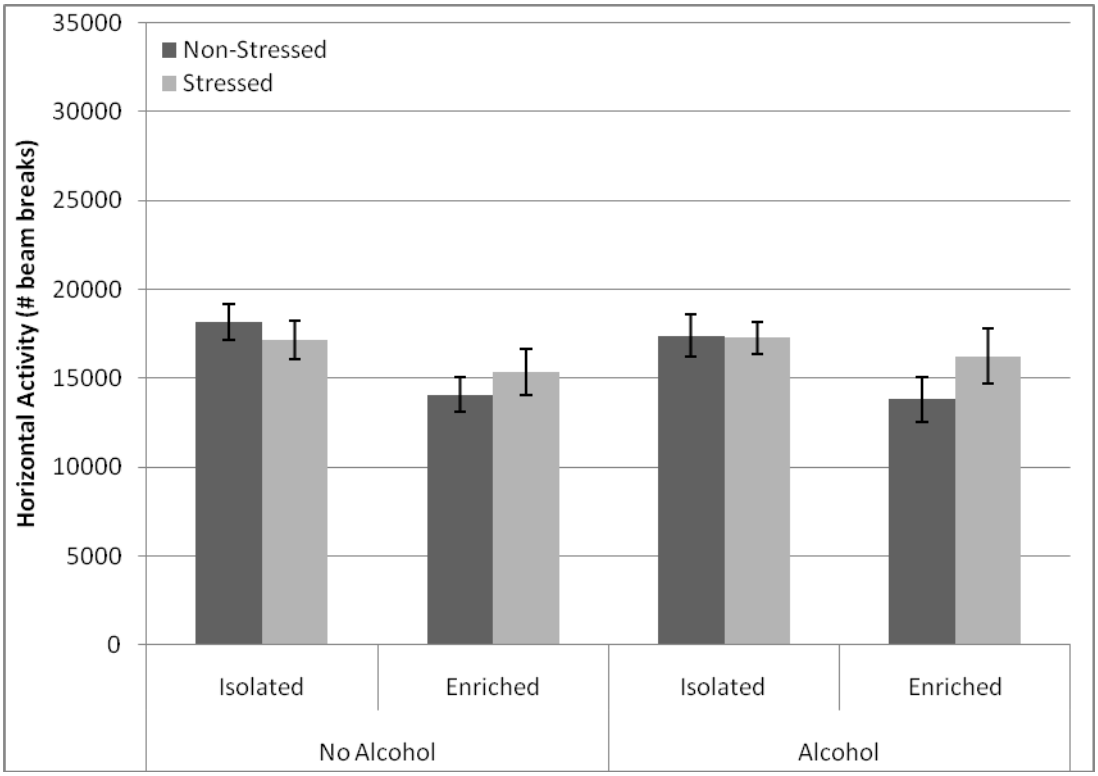


Figure 42 – Open Field Total Distance Traveled, Baseline Phase

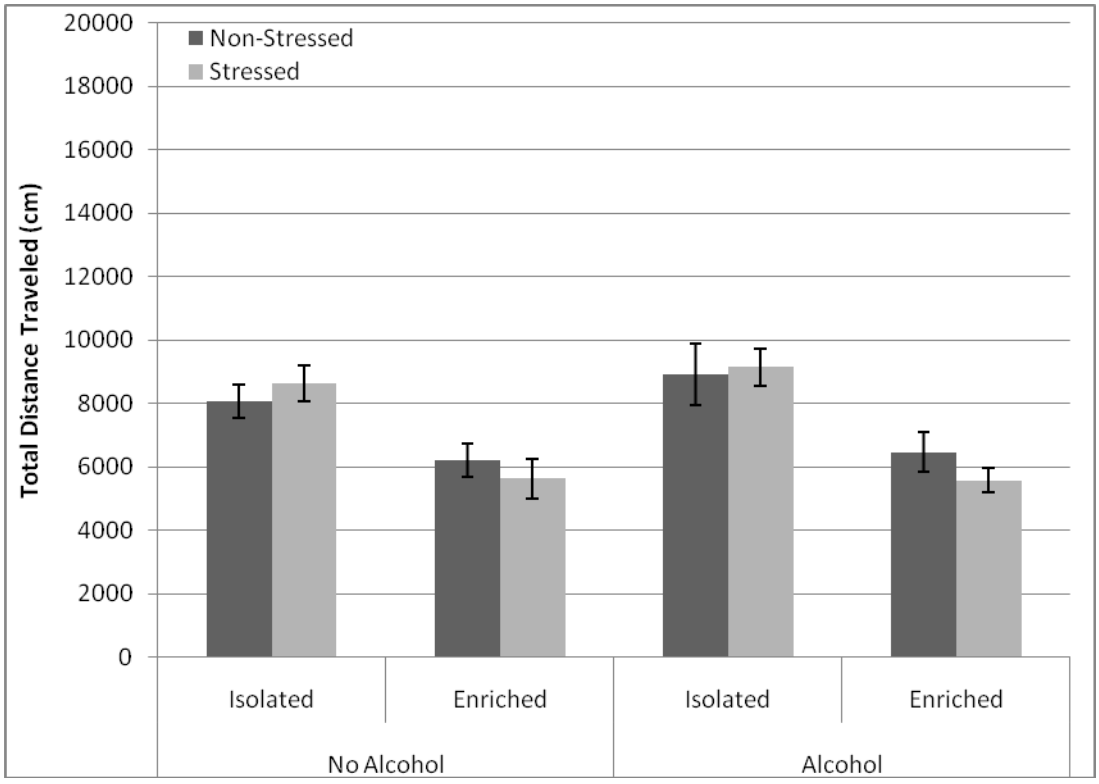


Figure 43 – Open Field Total Distance Traveled, 2BC Phase

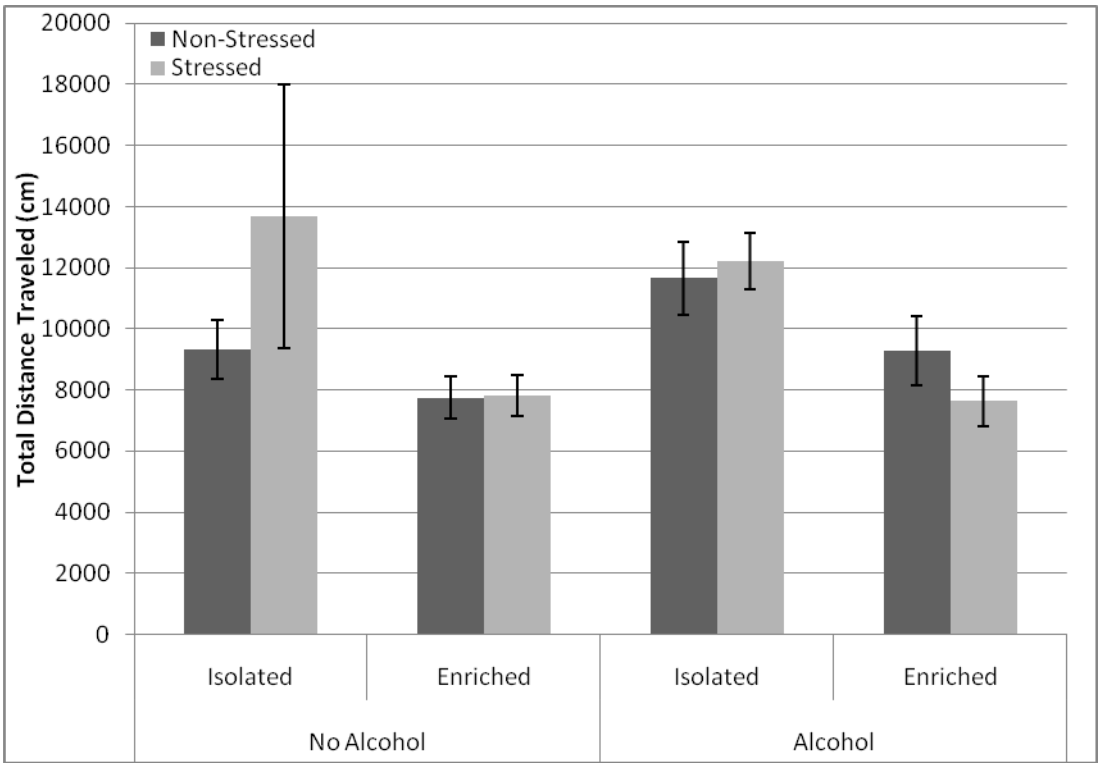


Figure 44 – Open Field Total Distance Traveled, LSA Phase

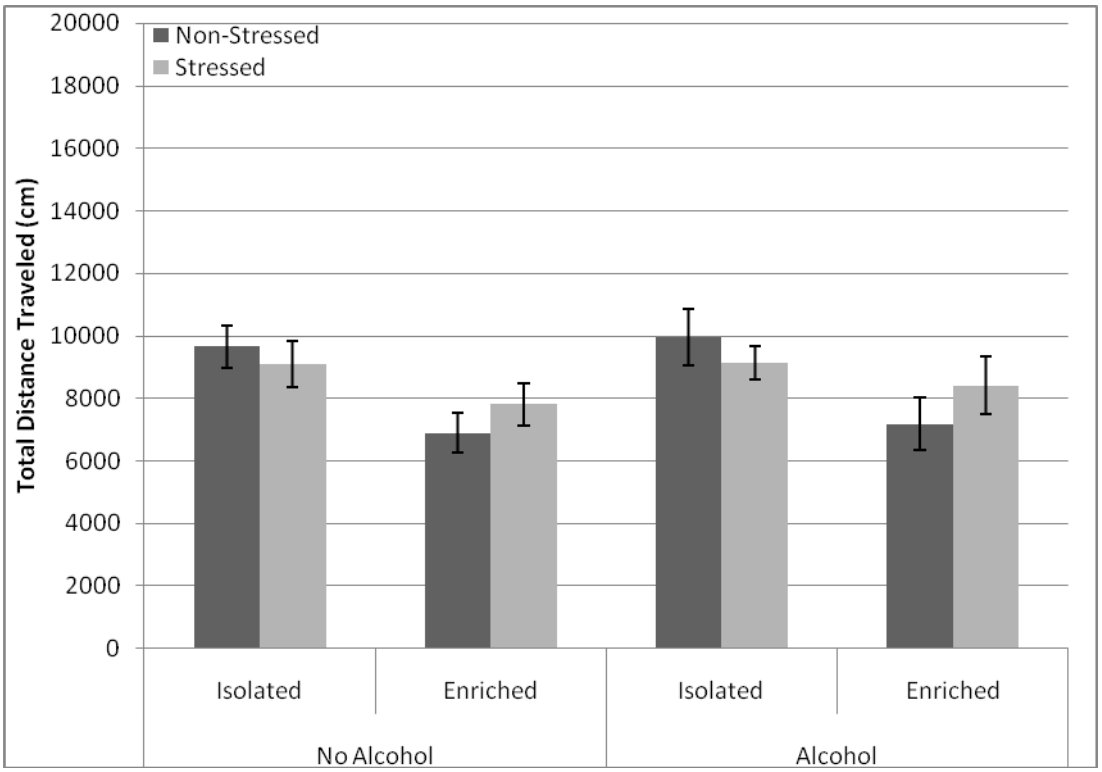


Figure 45 – Open Field Vertical Activity, Baseline Phase

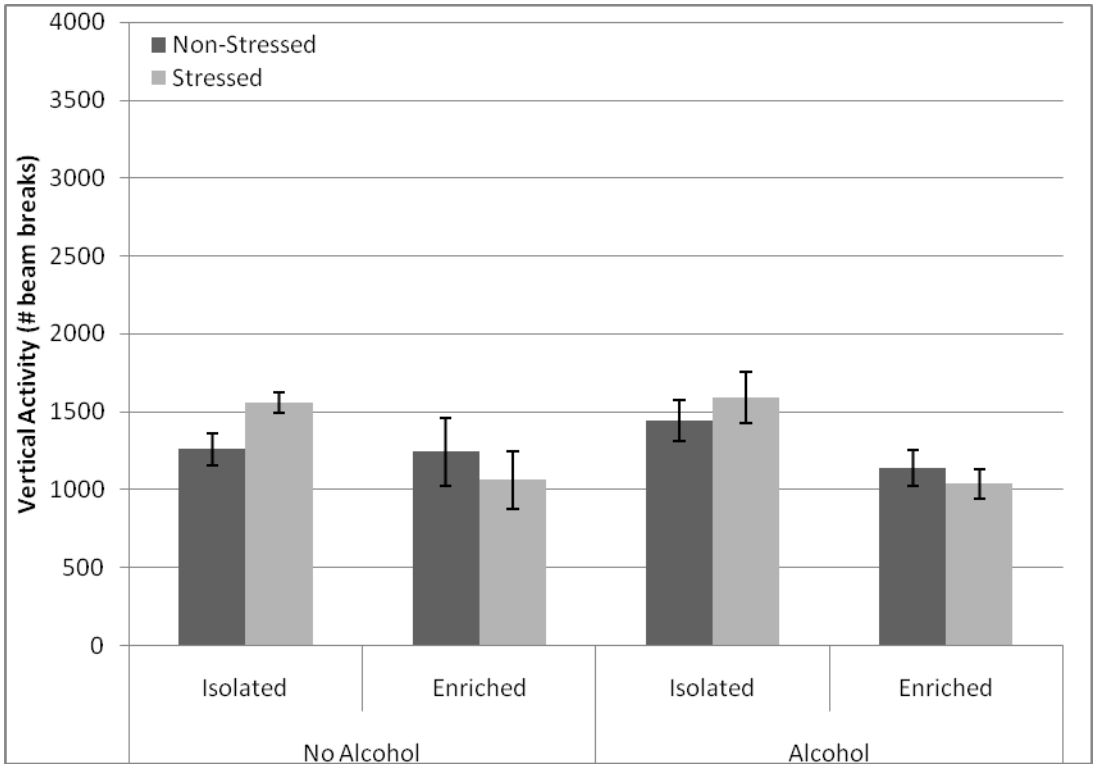


Figure 46 – Open Field Vertical Activity, Two-Bottle Choice Phase

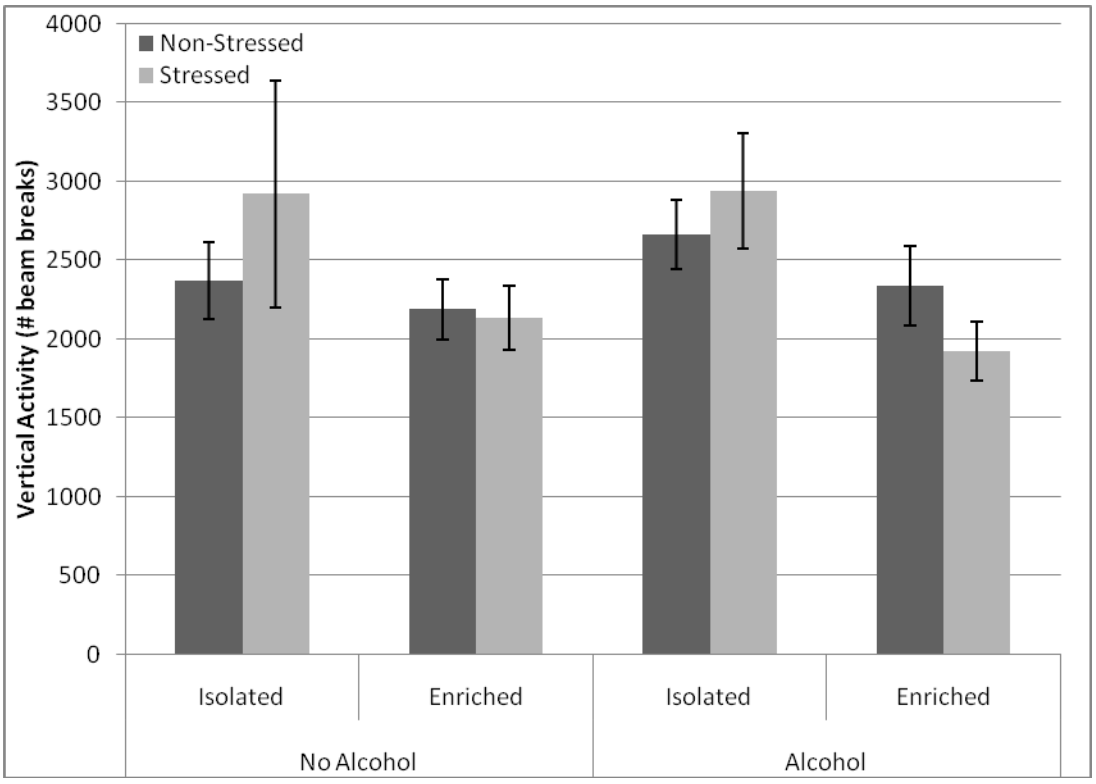


Figure 47 – Open Field Vertical Activity, Liquid Self-Administration Phase

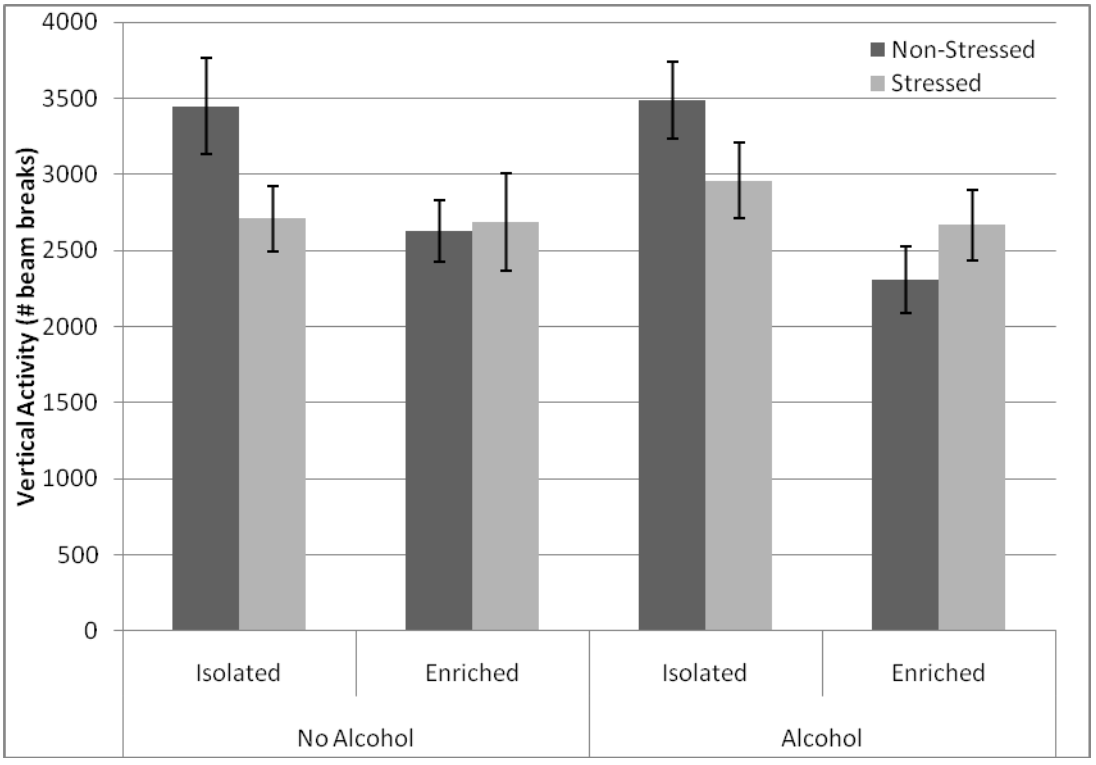


Figure 48 – Open Field Center Time, Baseline

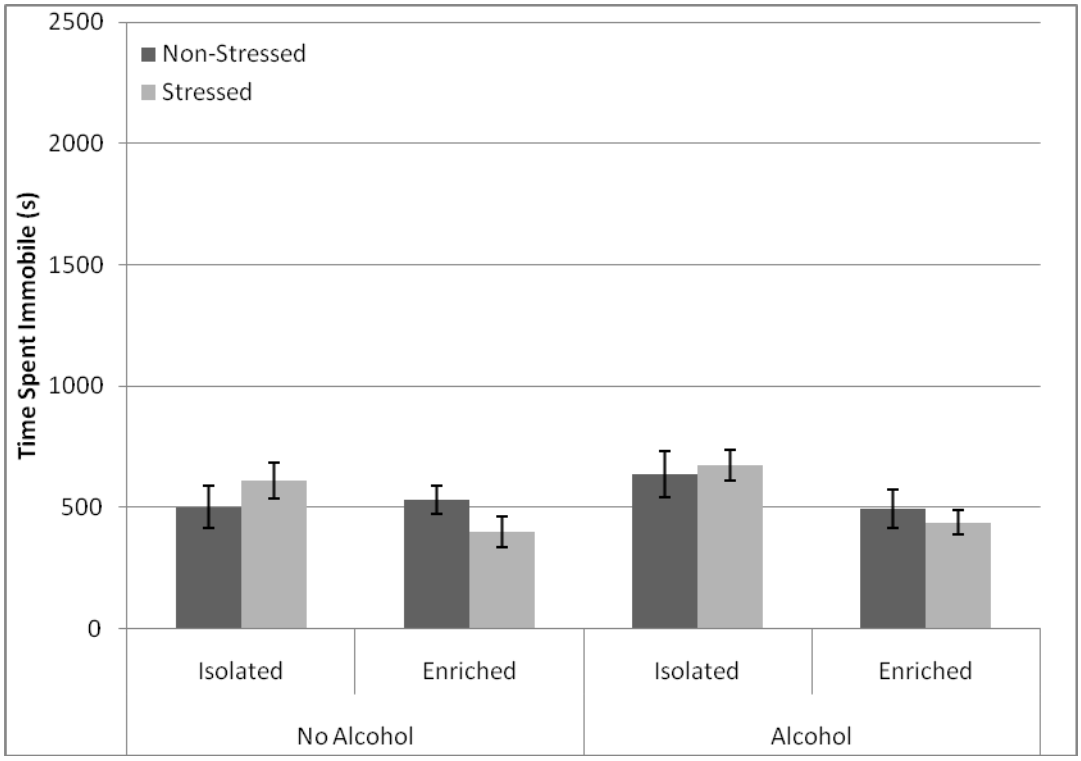


Figure 49 – Open Field Center Time, Two-Bottle Choice Phase

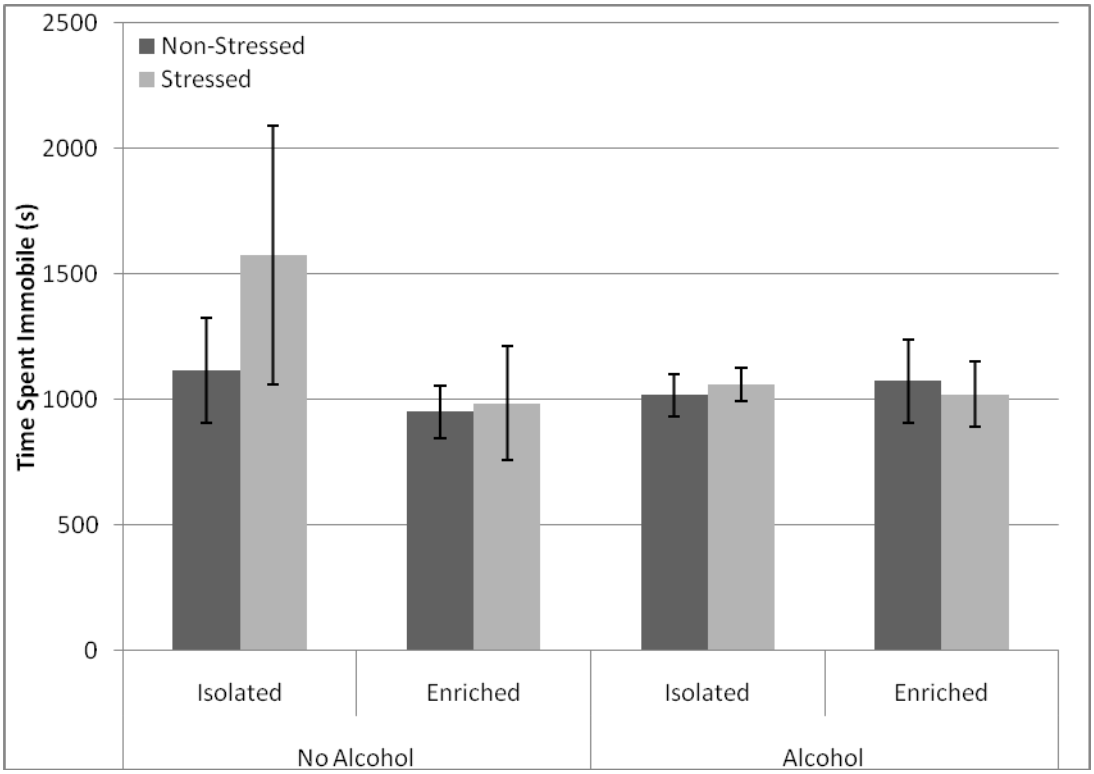


Figure 50 – Open Field Center Time, Liquid Self-Administration Phase

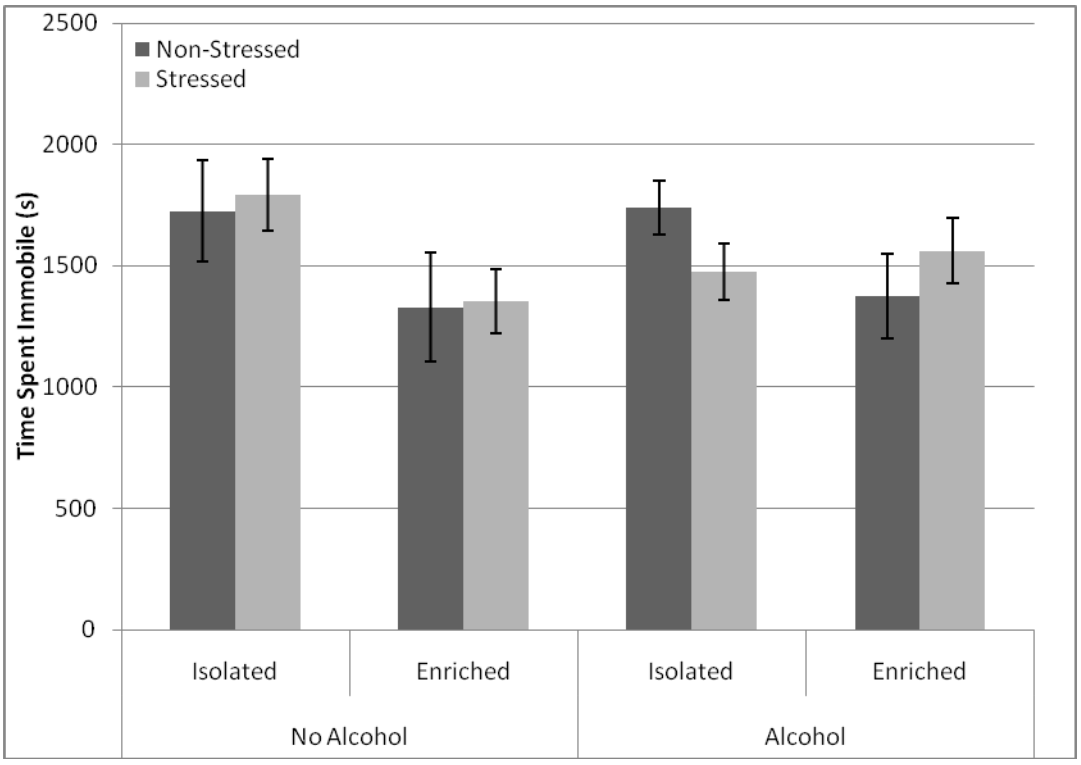


Figure 51 – Open Field Habituation, Baseline Phase

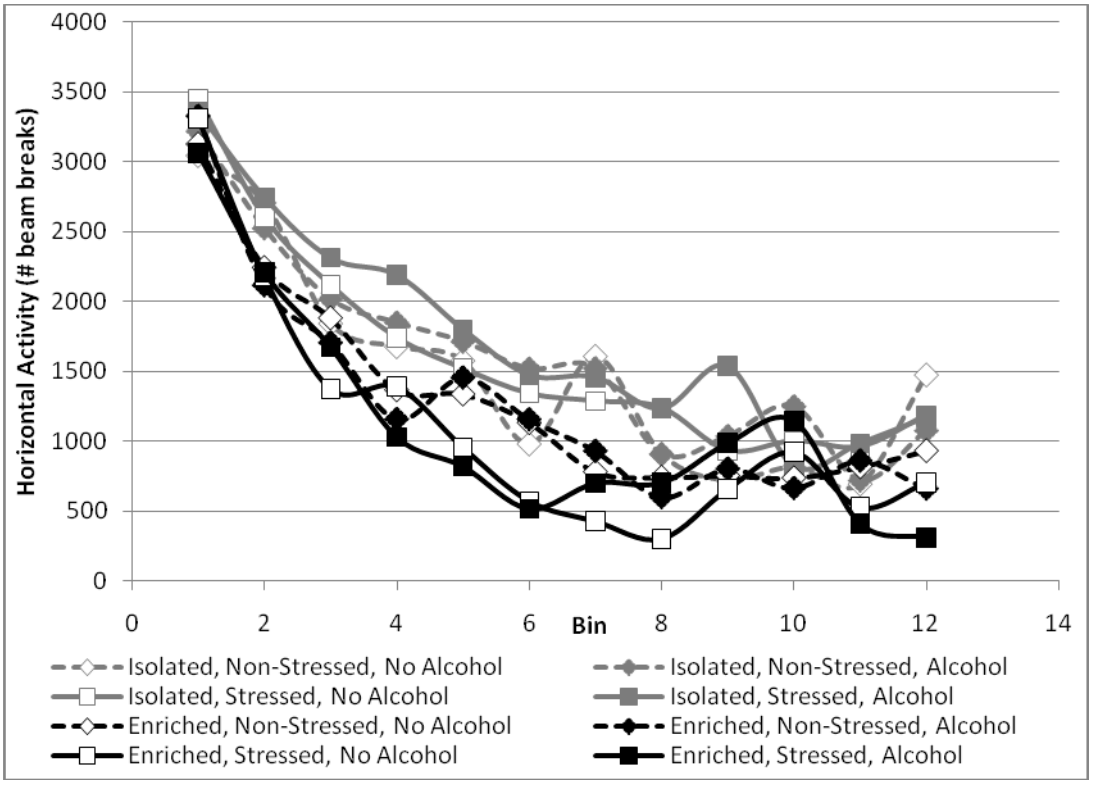


Figure 52 – Open Field Habituation, Two-Bottle Choice Phase

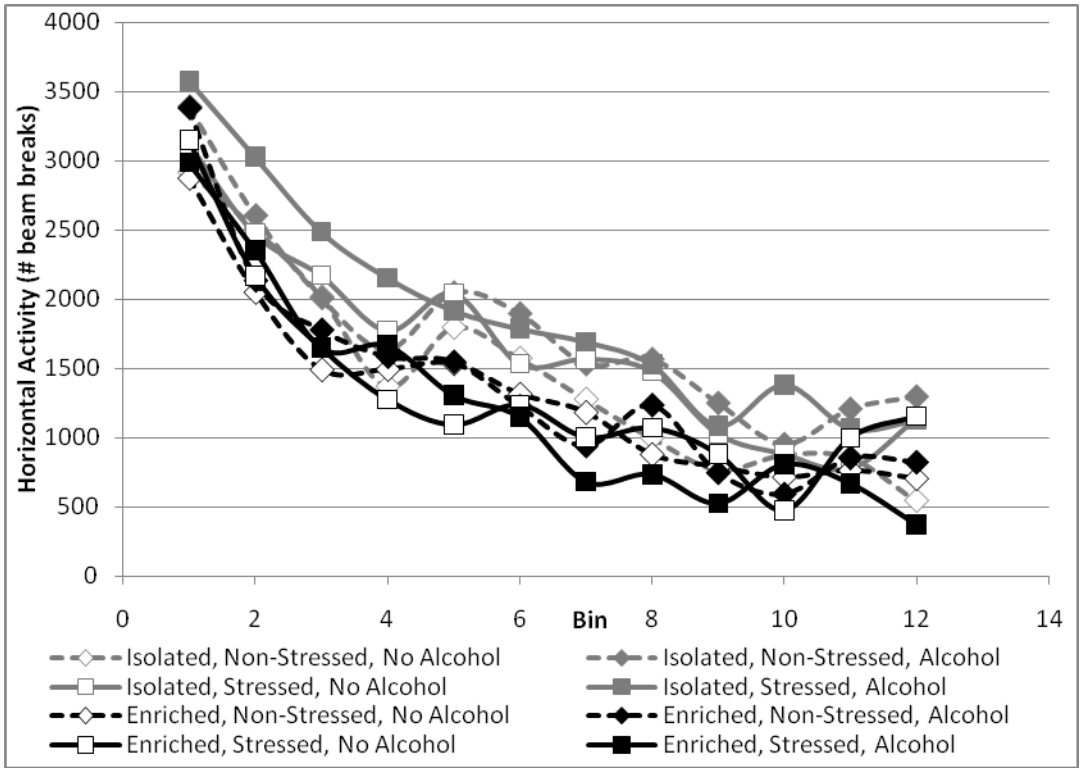


Figure 53 – Open Field Habituation, Liquid Self-Administration Phase

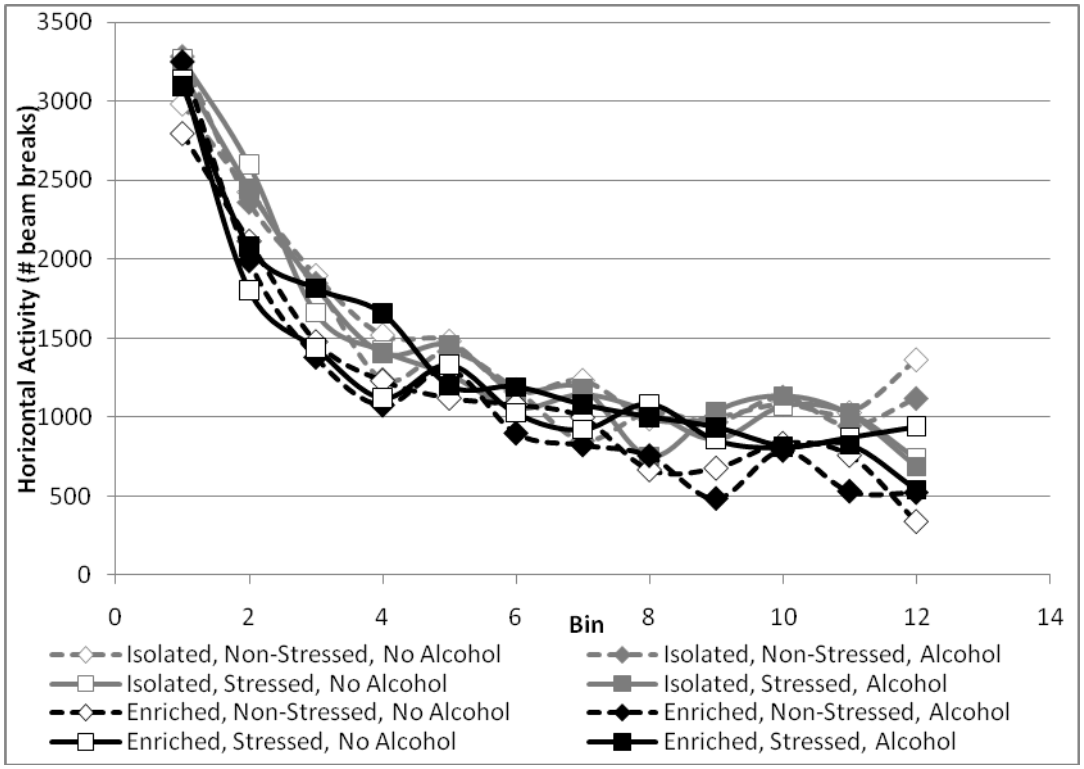


Figure 54 – Forced Swim Test, Total Time Spent Immobile during 2BC Phase

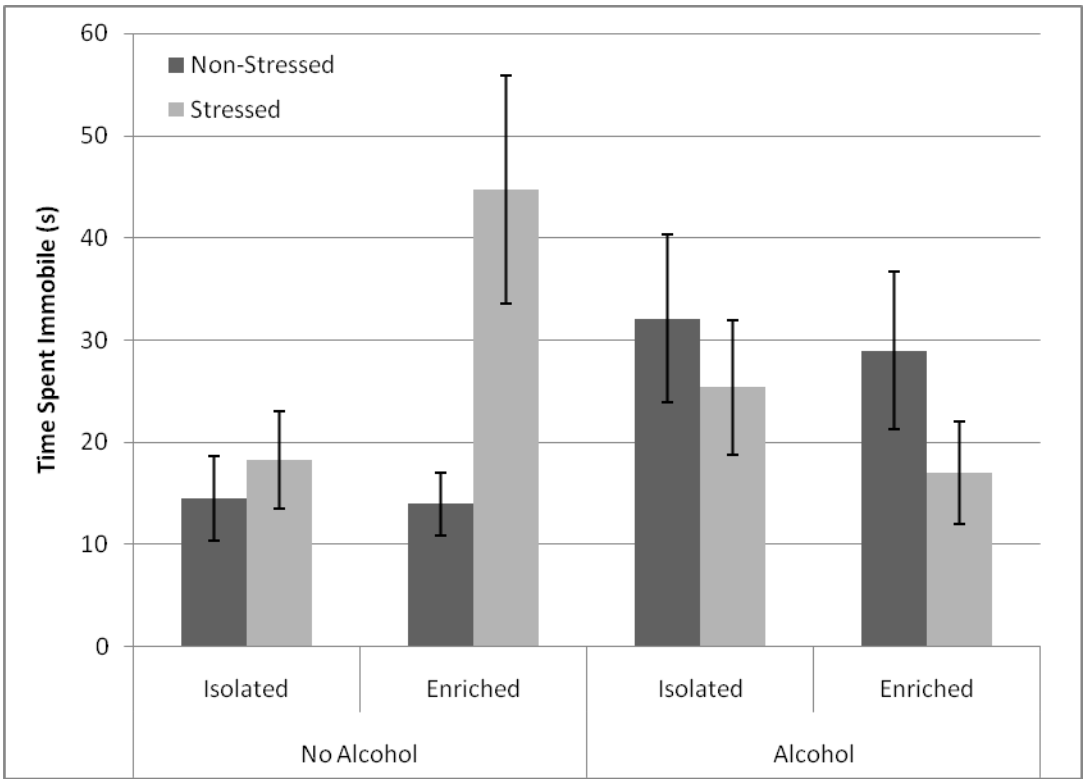


Figure 55 – Forced Swim Test, Number of Immobile Episodes during 2BC Phase

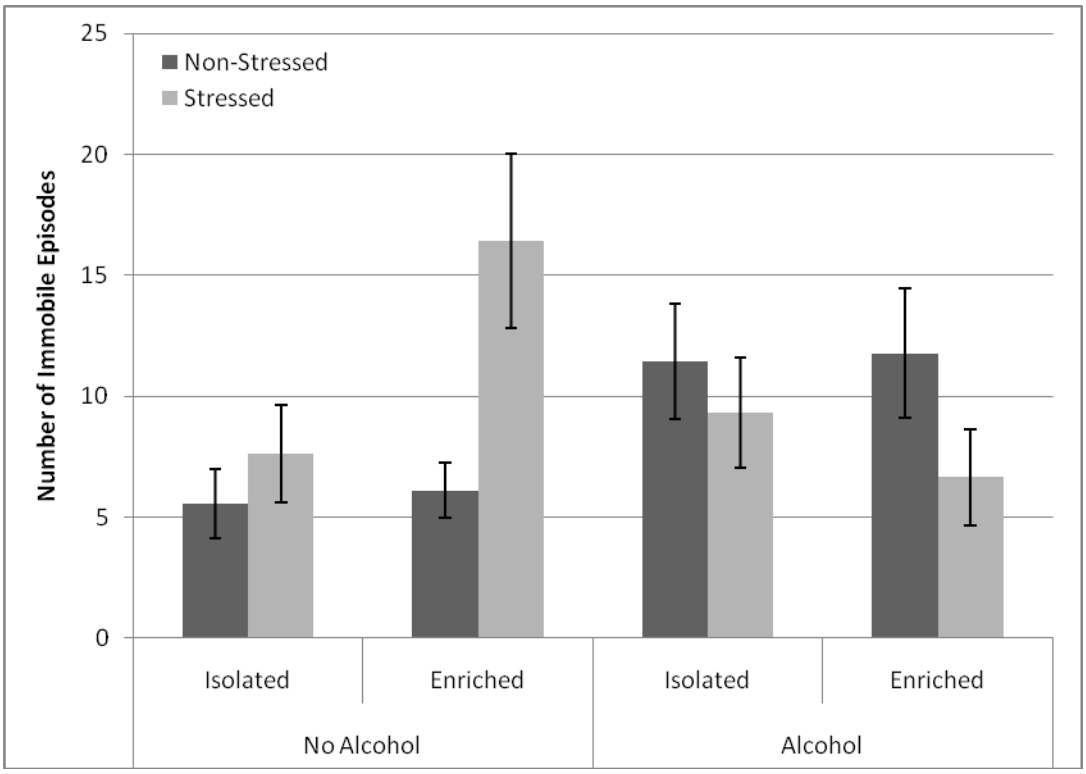


Figure 56 – Forced Swim Test, Total Time Spent Immobile during LSA Phase

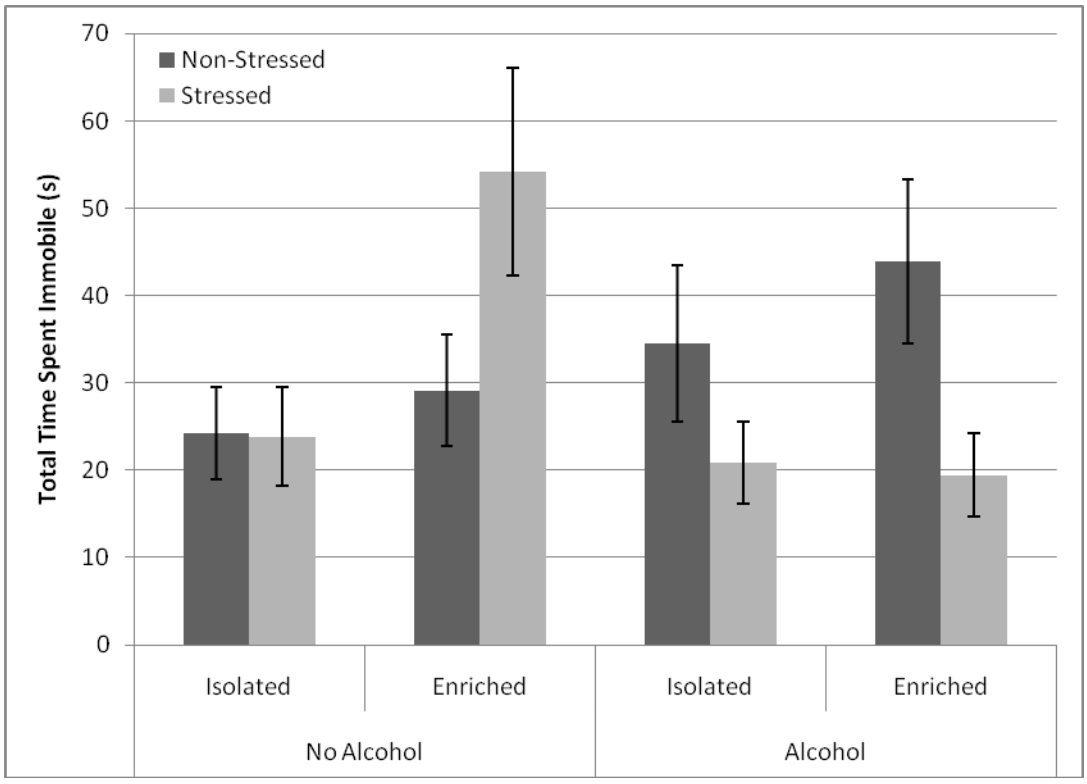


Figure 57 – Forced Swim Test, Number of Immobile Episodes during LSA Phase

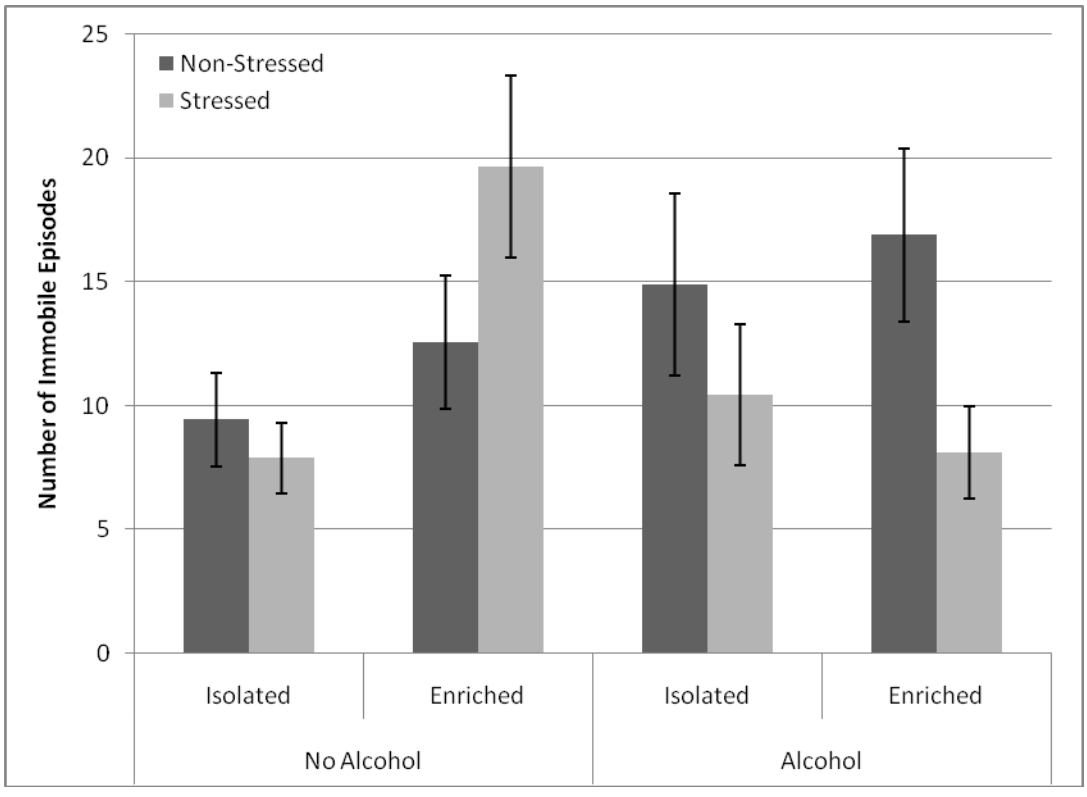


Figure 58 – Dopamine Concentration in Nucleus Accumbens

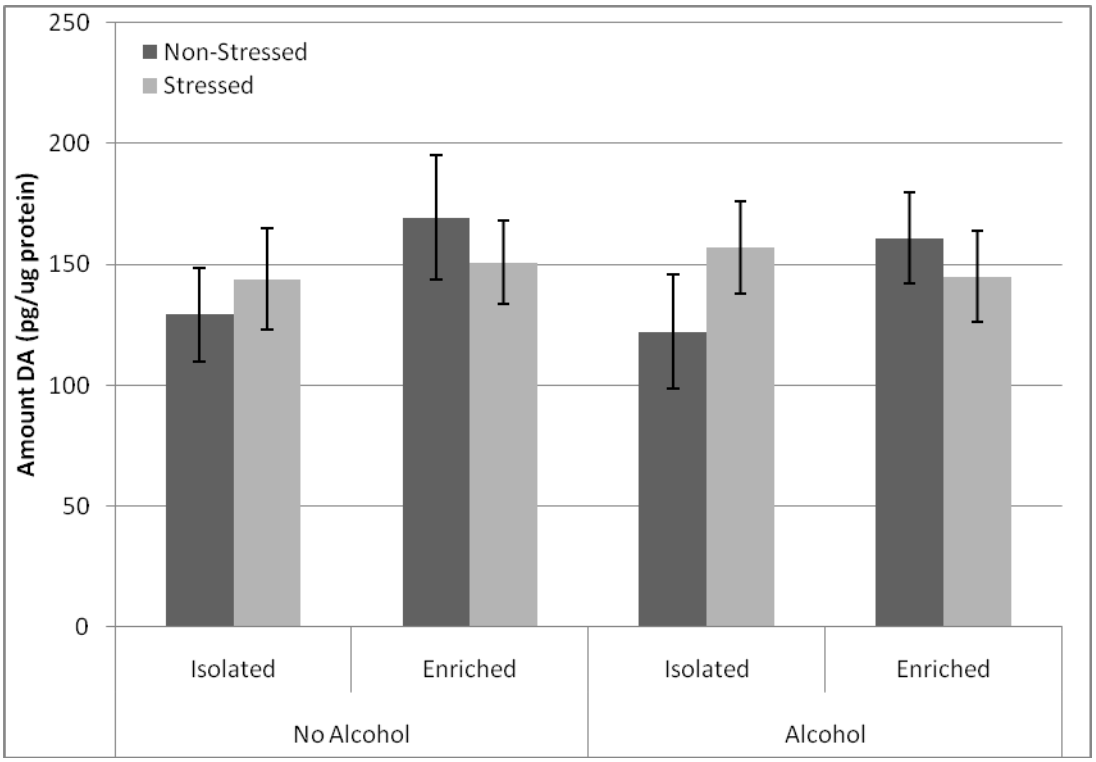


Figure 59 – 3,4-Dihydroxyphenylacetic Acid Concentration in Nucleus Accumbens

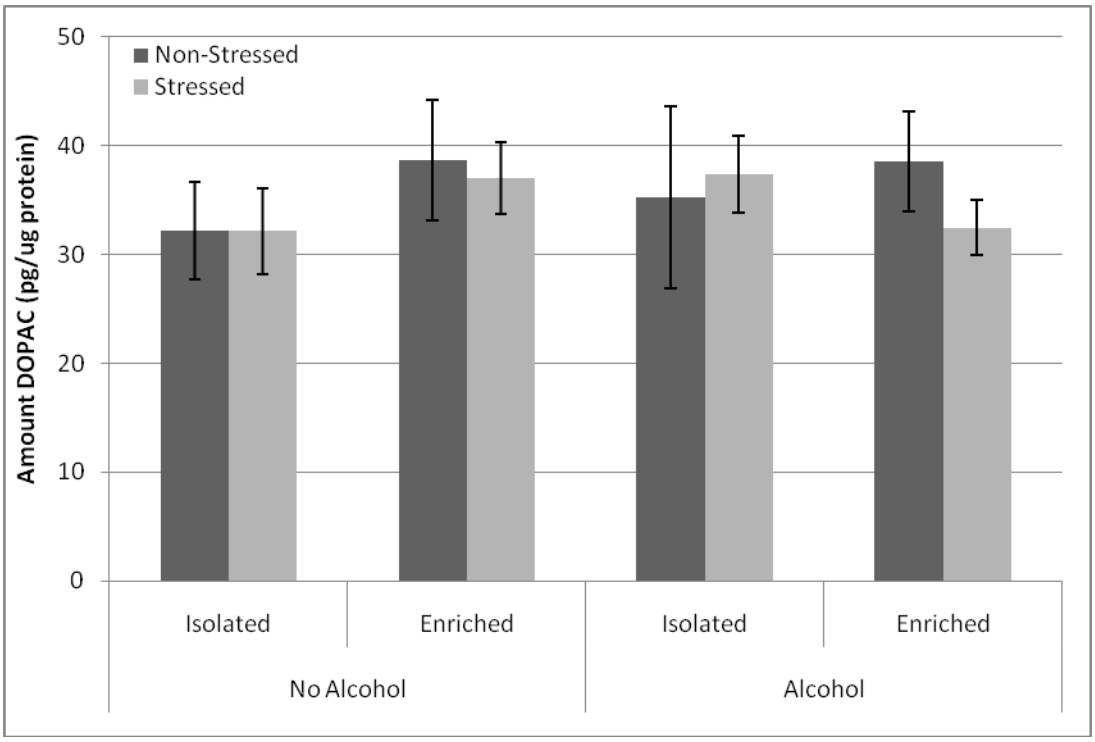


Figure 60 – Homovanillic Acid Concentration in Nucleus Accumbens

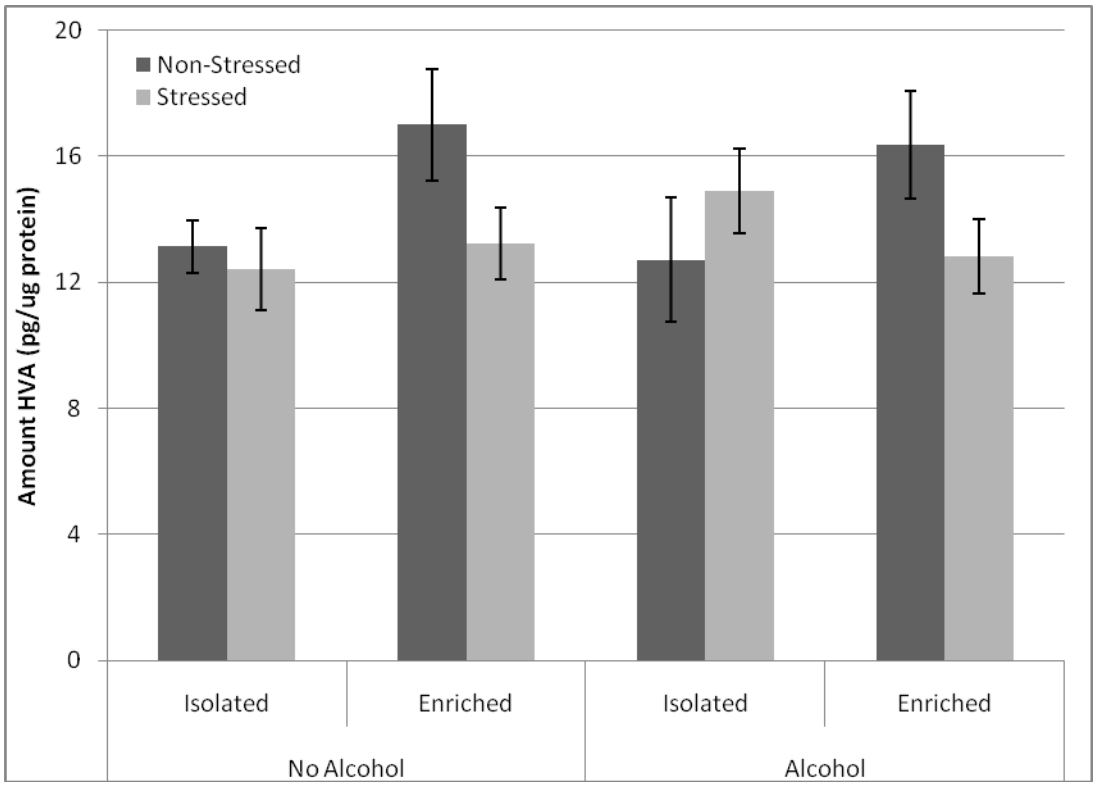


Figure 61 – Norepinephrine Concentration in the Nucleus Accumbens

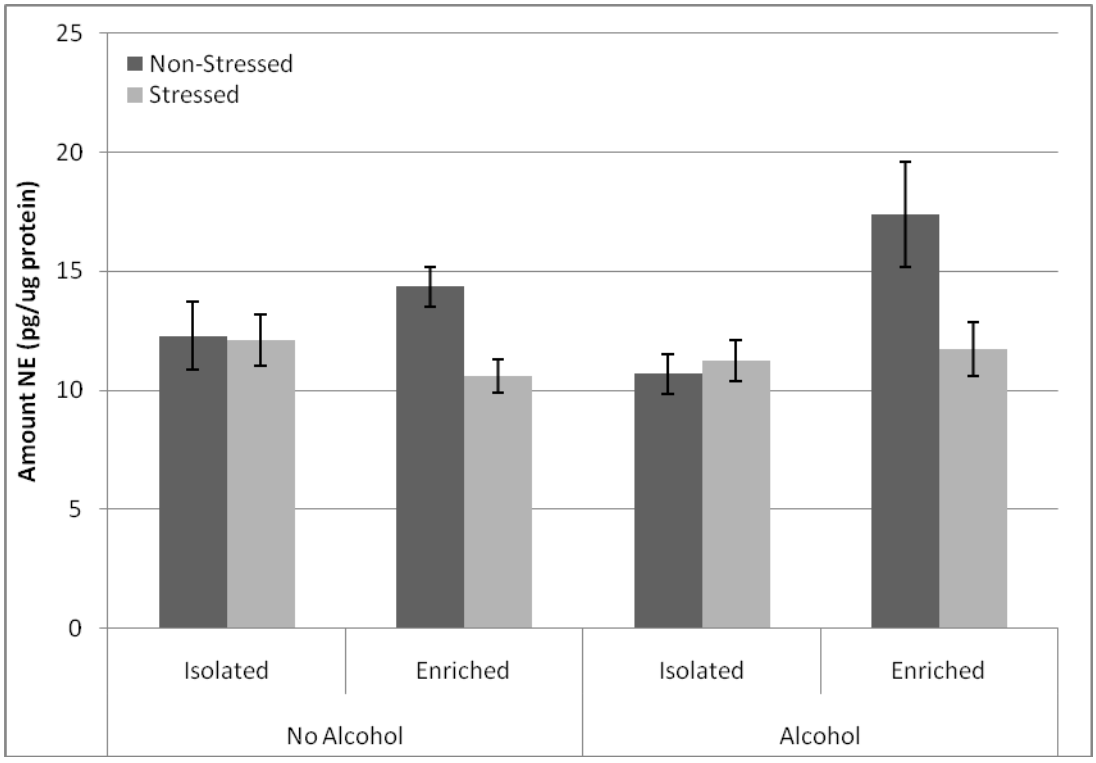


Figure 62 – Serotonin Concentration in Nucleus Accumbens

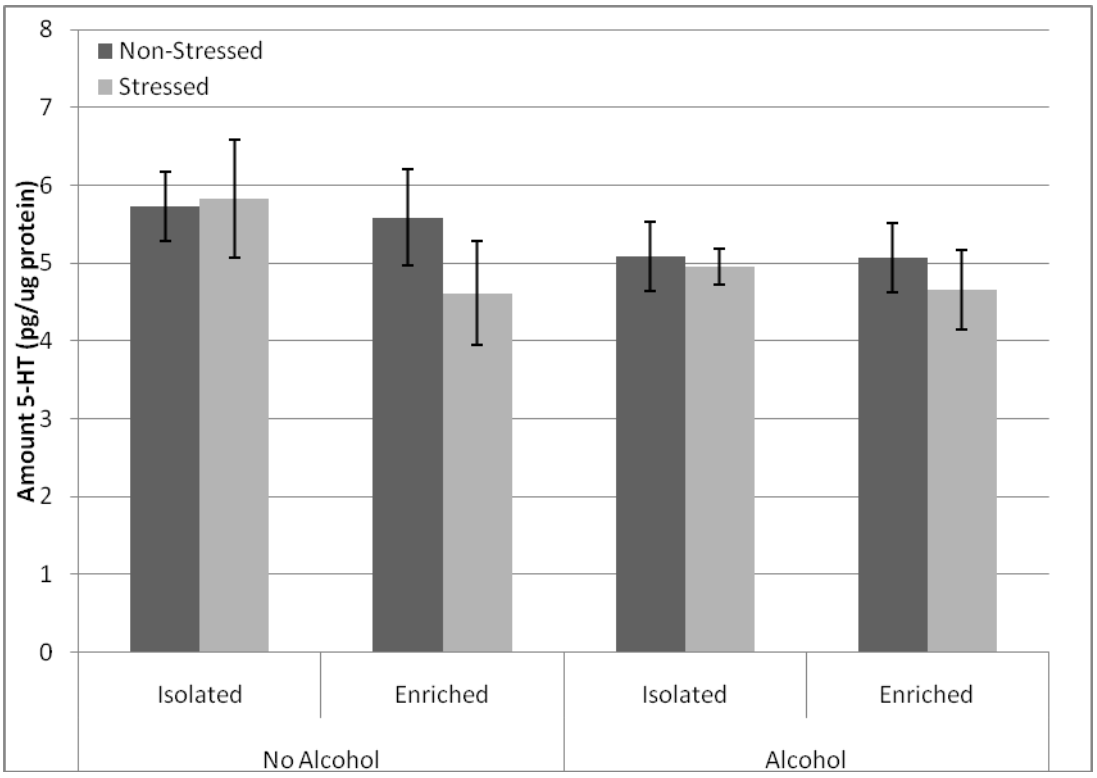


Figure 63 – 5-Hydroxyindoleacetic Acid Concentration in Nucleus Accumbens

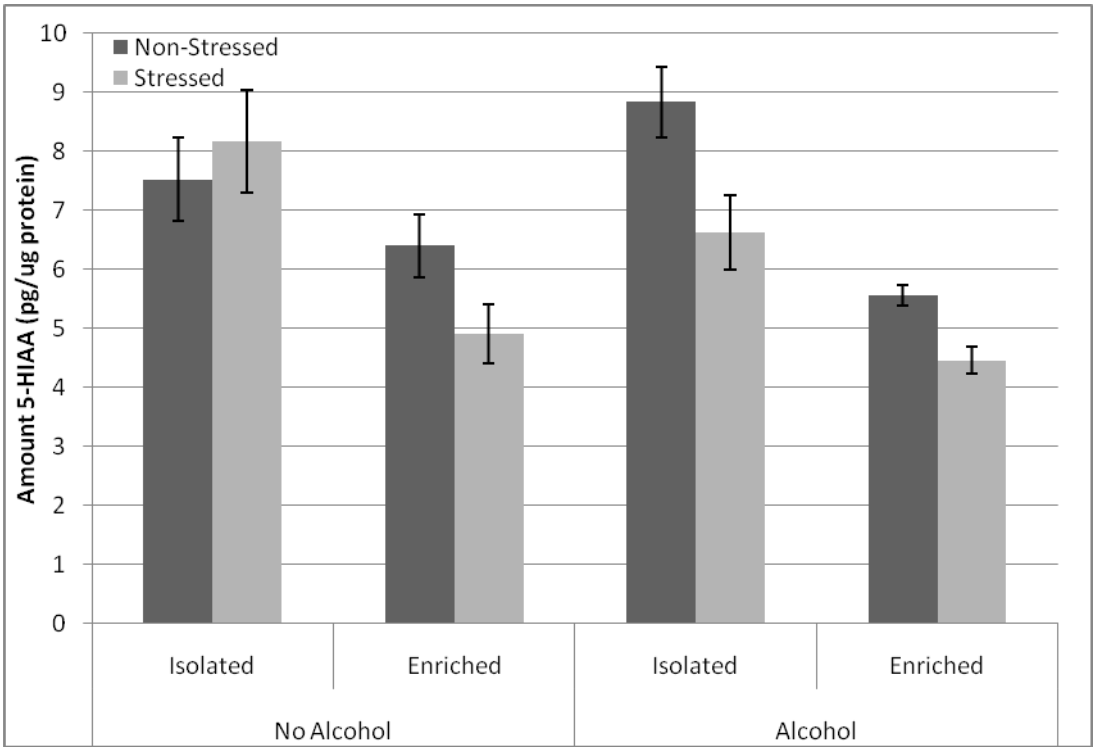


Figure 64 – Dopamine Concentration in Ventral Tegmental Area

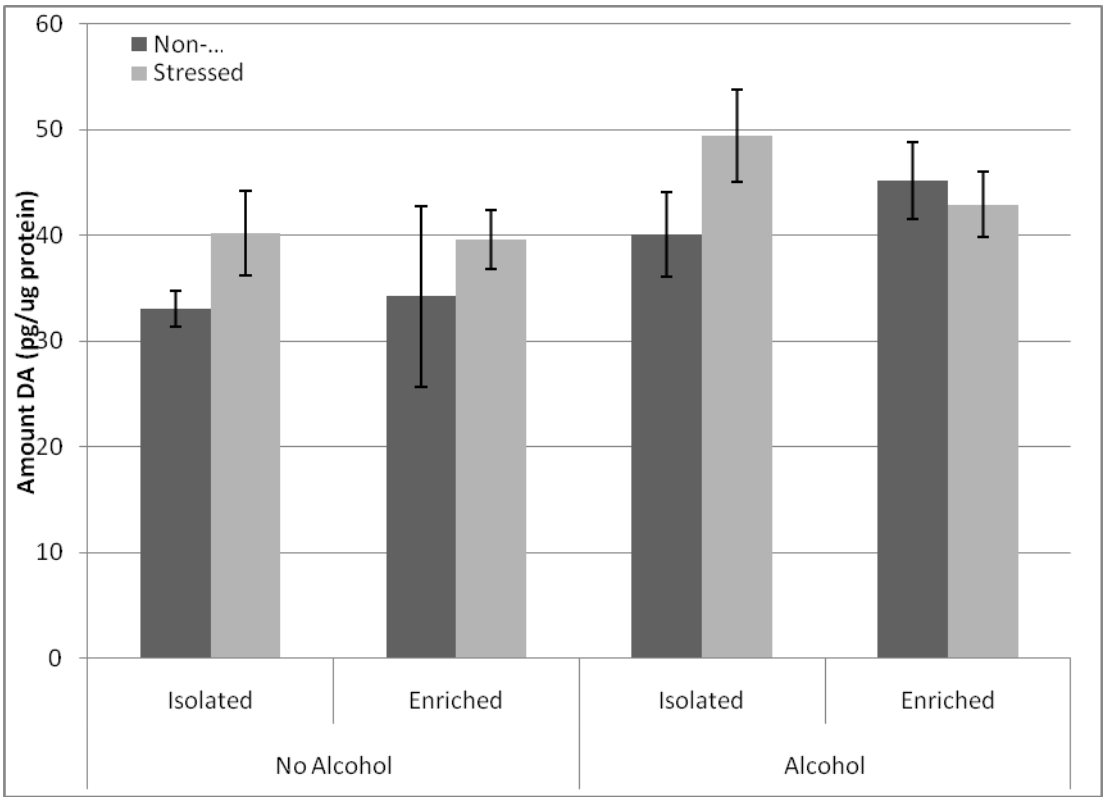


Figure 65 – 3,4-Dihydroxyphenylacetic Acid Concentration in Ventral Tegmental Area

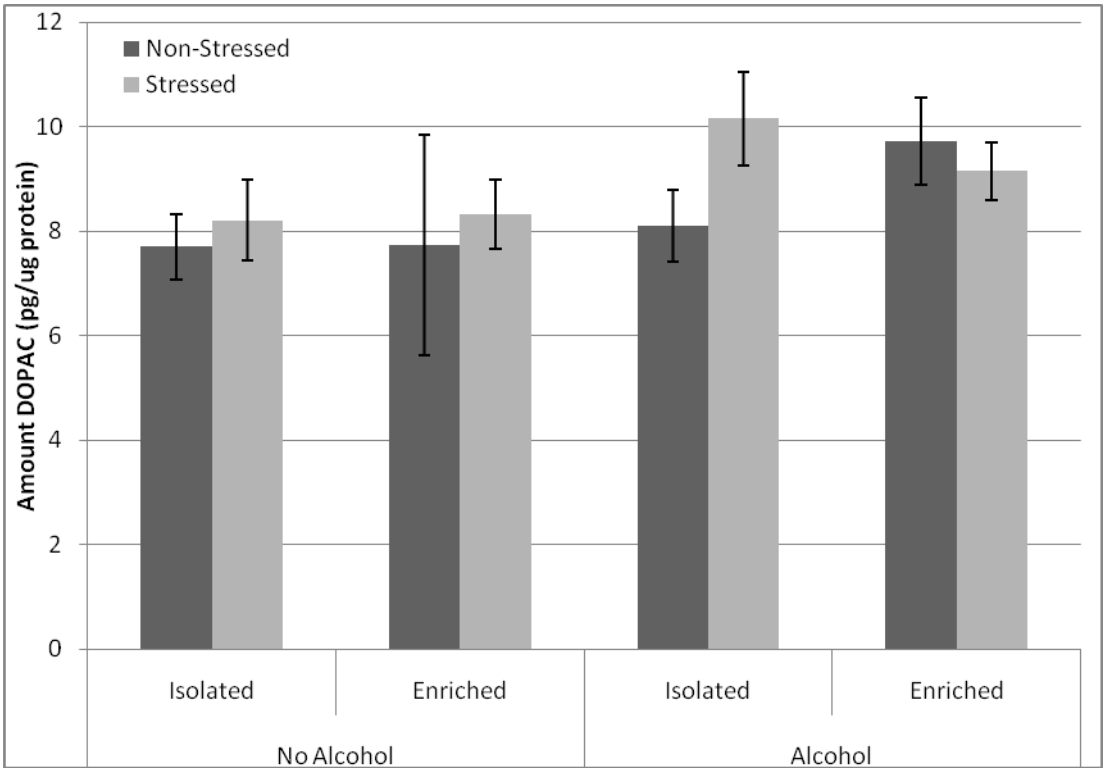


Figure 66 – Homovanillic Acid Concentration in Ventral Tegmental Area

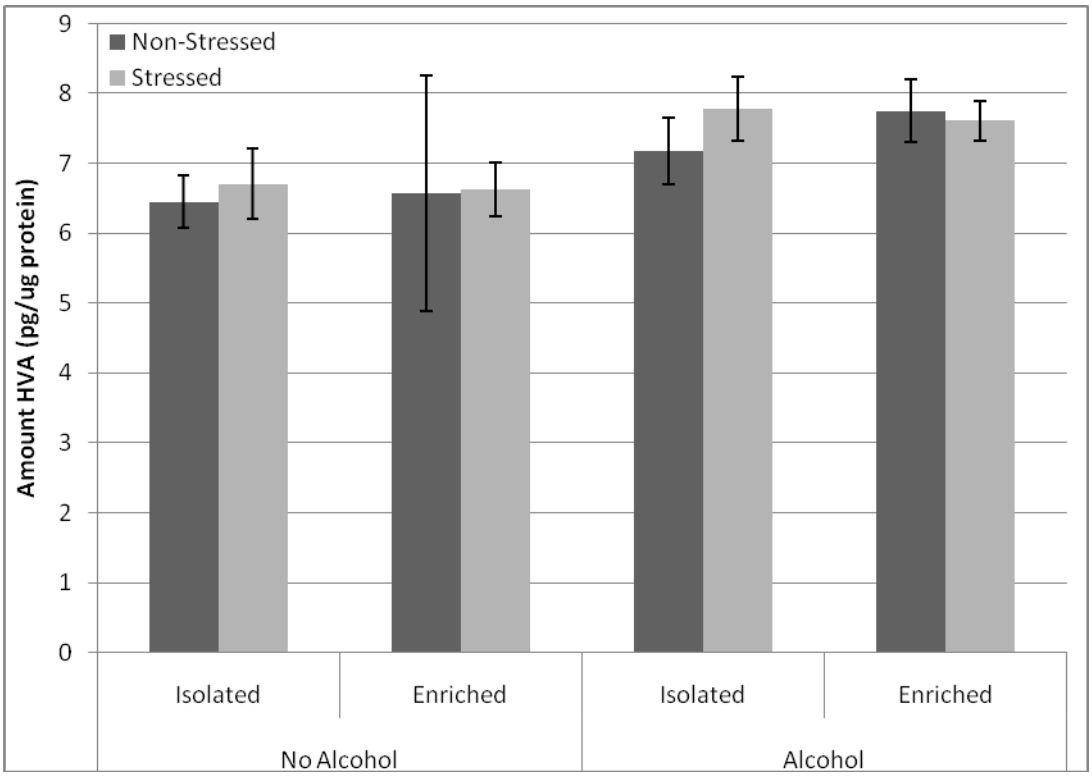


Figure 67 – Norepinephrine Concentration in Ventral Tegmental Area

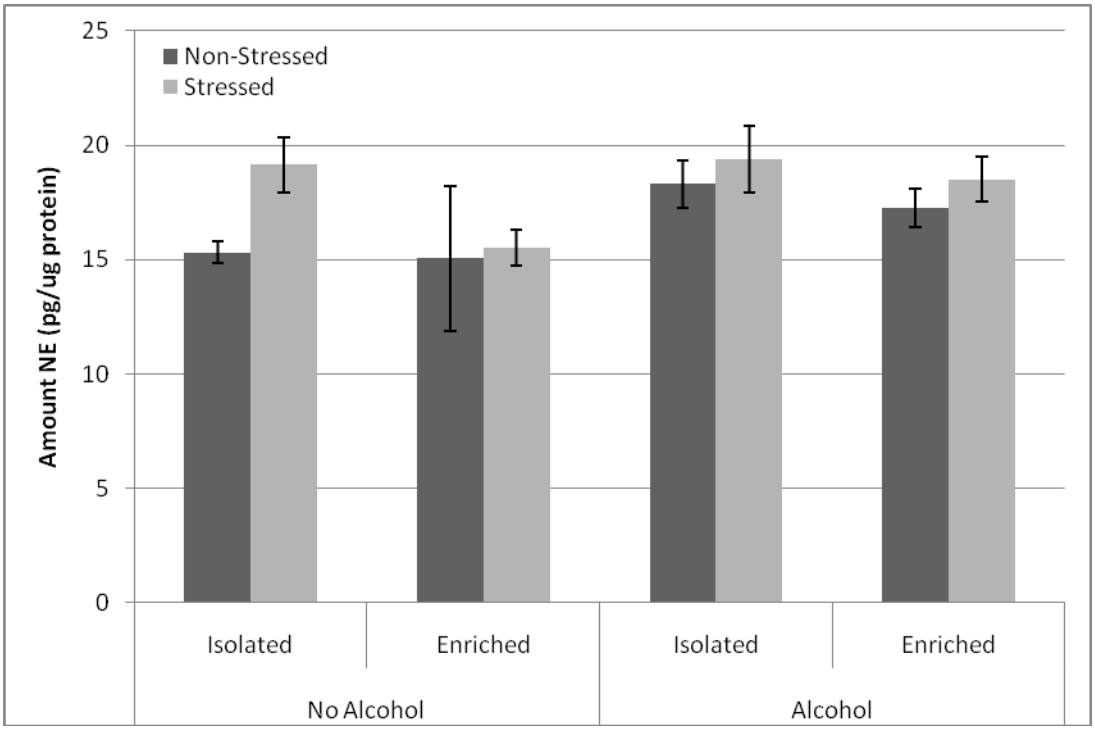


Figure 68 – Serotonin Concentration in Ventral Tegmental Area

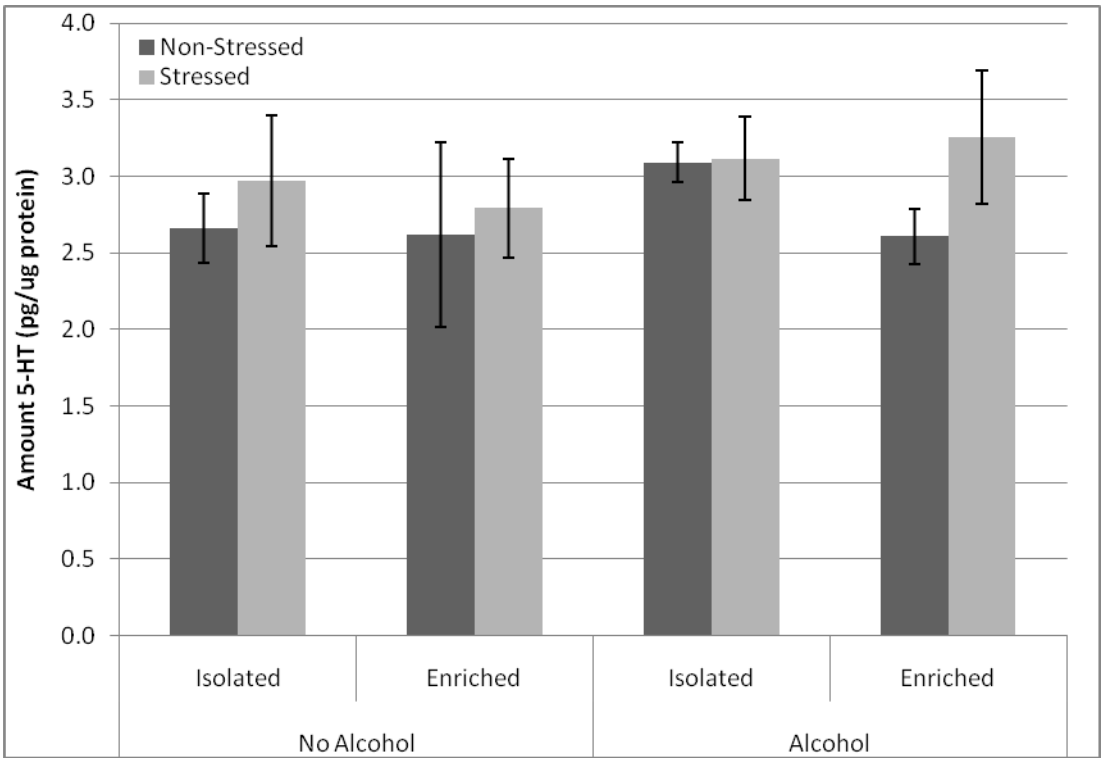


Figure 69 – 5-Hydroxyindoleacetic Acid Concentration in Ventral Tegmental Area

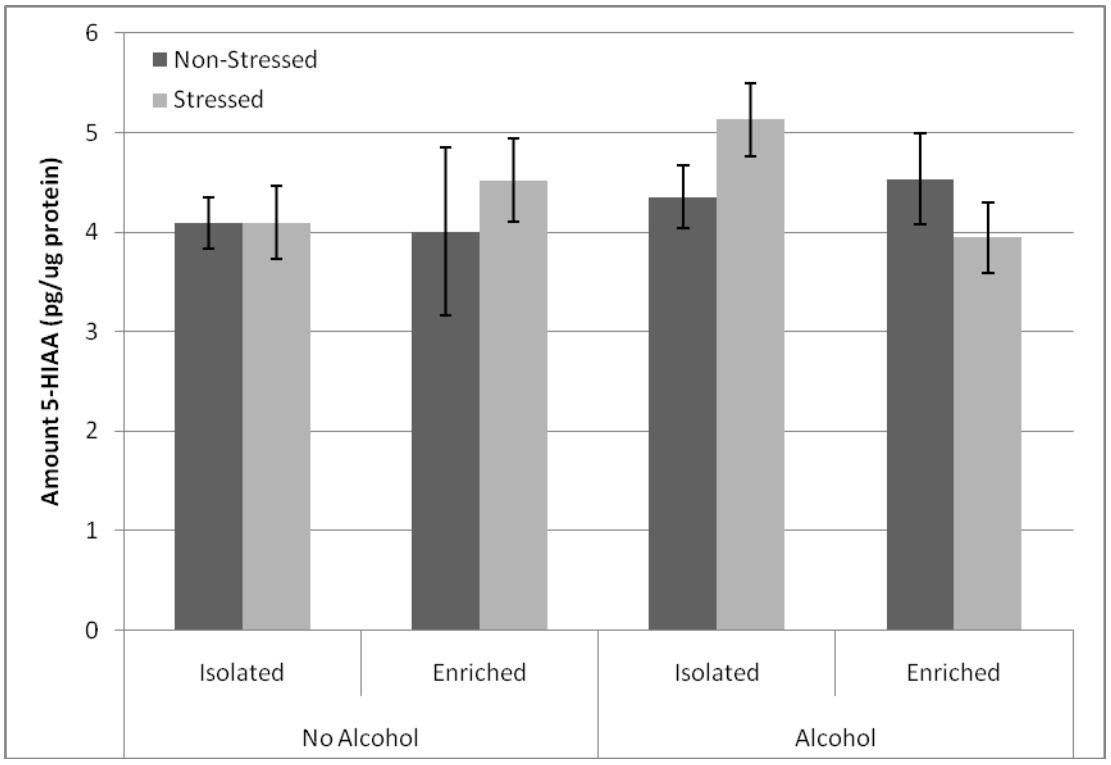


Figure 70 – Dopamine Concentration in Prefrontal Cortex

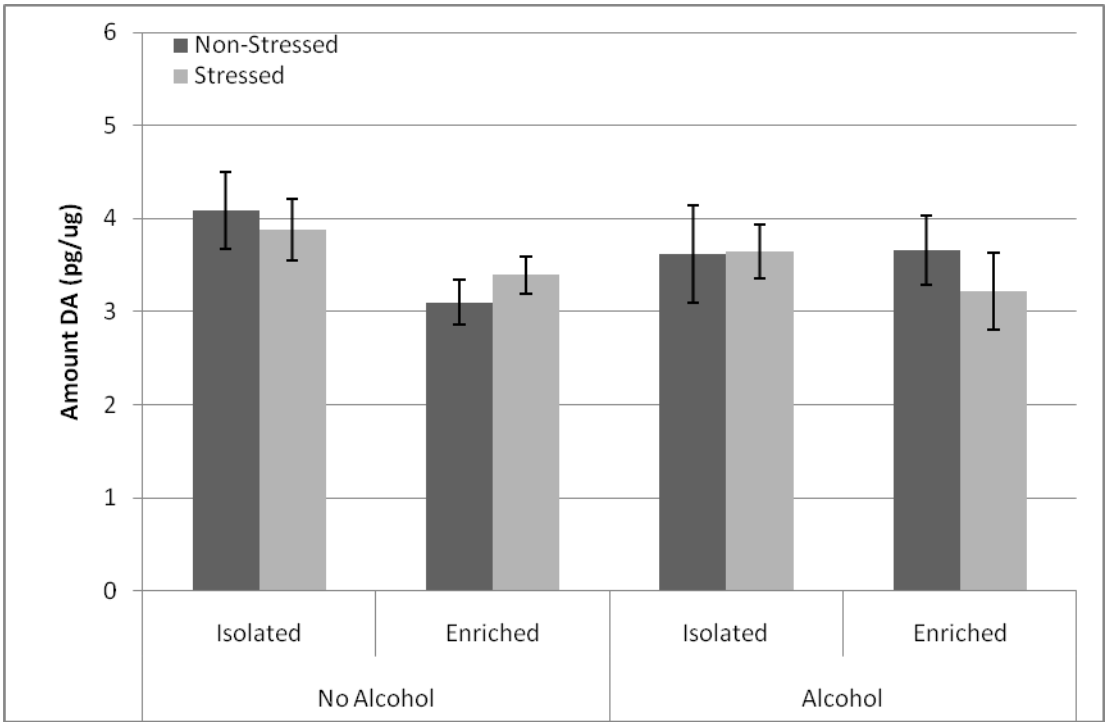


Figure 71 – 3,4-Dihydroxyphenylacetic Acid Concentration in Prefrontal Cortex

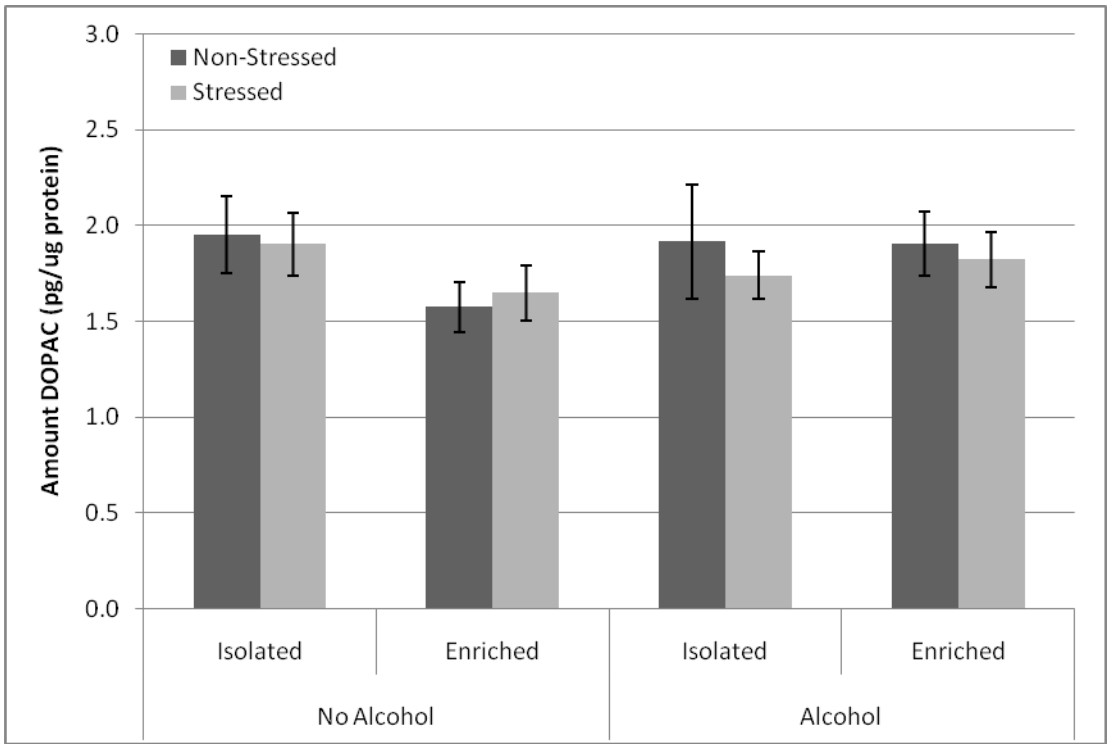


Figure 72 – Homovanillic Acid Concentration in Prefrontal Cortex

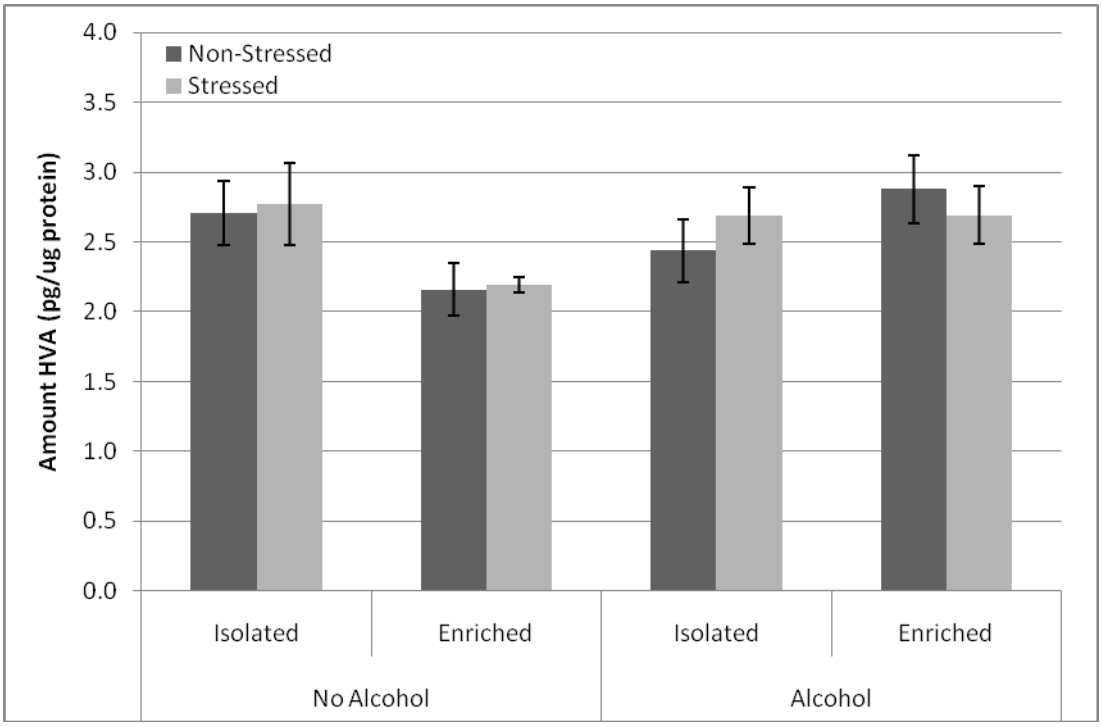


Figure 73 – Norepinephrine Concentration in Prefrontal Cortex

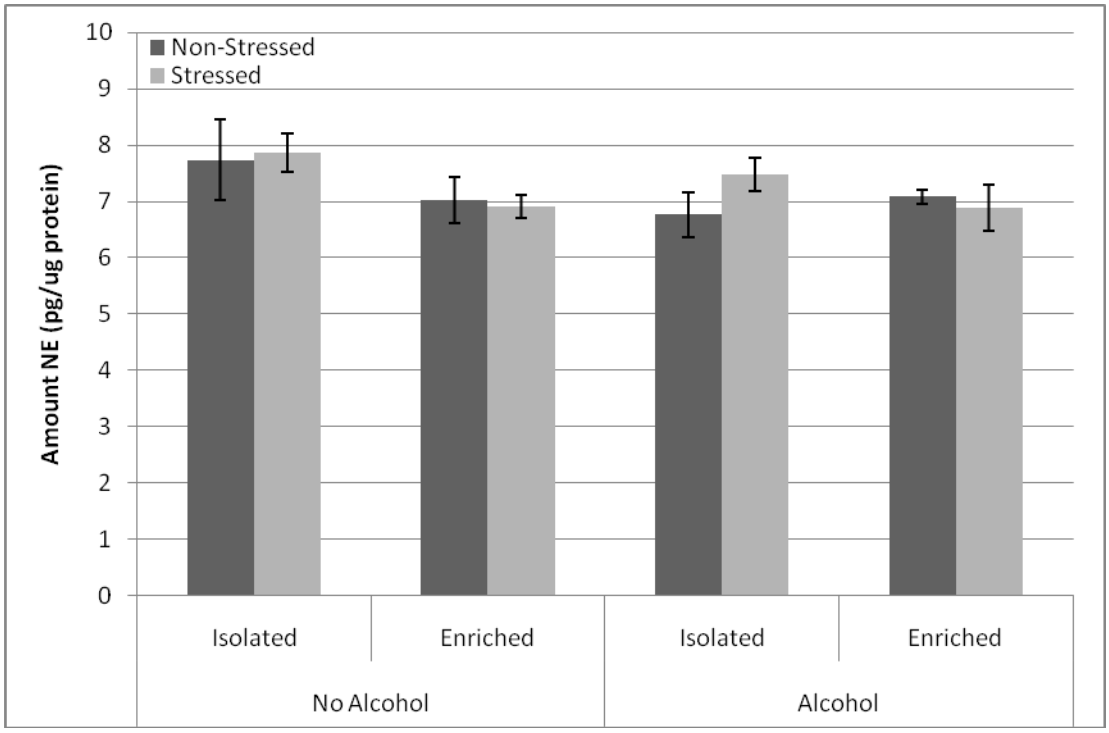


Figure 74 – Serotonin Concentration in Prefrontal Cortex

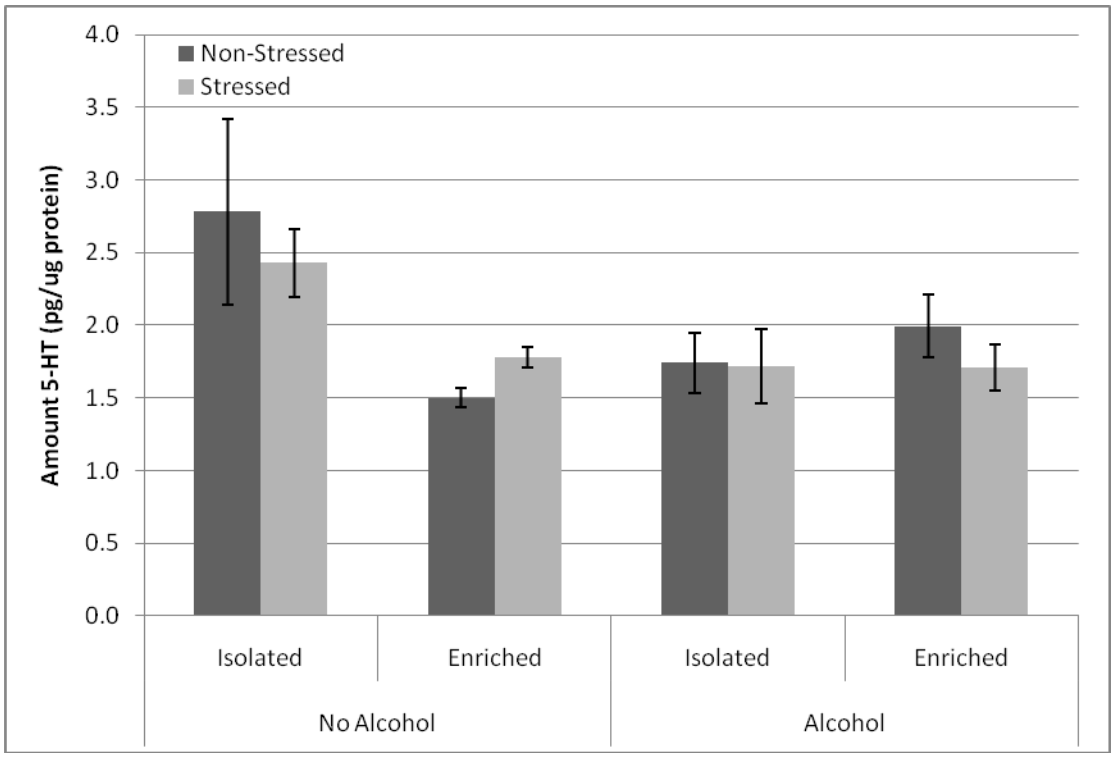


Figure 75 – 5-Hydroxyindoleacetic Acid Concentration in Prefrontal Cortex